CHARACTERIZATION OF IMMUNE SERA IN AMEBIASIS

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C E R T I F I C A T E

I certify that the work presented in this thesis has been carried out in my laboratory by Mr. Ali Cher. Part of this work was also done by him at the State Serum Institute, Copenhagen, Denmark. The thesis is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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D E D I C A T I O N

Dedicated to the memory of my dear father who was a constant source of inspiration; I also dedicate this work to my mother, brothers, sisters and to my wife.

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A B S T R A C T

Characterization of anti-*Entamoeba histolytica* antibodies were carried out in sera samples obtained from experimental animals and, also from amebiasis patients. *E. histolytica* (strain HM: 200) was grown axenically in TP-S-1-monophasic medium for 96 hrs at 37°C and then subsequently harvested, pooled, washed and utilized for making water soluble antigenic extracts. The antigenic extracts were made by mechanical disruption of the amoebae. Animals were immunized with water soluble antigen in combination with alumina adjuvant. During immunization, the animals were also given a booster injection in the 4th week. Antibodies were detected to assess the humoral immune responses by making use of several antigen-antibody tests. The tests used for this purpose included precipitin, indirect haemagglutination, immunodiffusion, immunoelectrophoresis, fluorescent antibody, latex agglutination and bentonite slide flocculation. The highest dilution of antigen which gave a positive reaction against rabbit antiserum was 1:256. The optimal antigen dilution of 1:256 was used as the standard concentration in all the antigen-antibody reactions. Serum antibody titres in the range of 1:160 in bentonite slide flocculation tests appeared to be significant. Highest dilutions of the immunised serum giving a positive IHA and FA tests were found to be 1:4096 and 1:8192 respectively. Mostly, five precipitin bands were detectable in rabbit antisera samples in diffusion and immunoelectrophoresis tests. The indirect haemagglutination test was the most sensitive test for the

detection of antibodies in immunised sera samples. Other test sensitivities appeared in the order of precipitation, fluorescent antibody, and skin hypersensitivity reactions.

In the post immunization period a primary antibody was obtainable in the first four weeks. This was followed by a very strong secondary response, following a booster injection in the fourth week. Subsequently, the level of antiamebic antibodies increased greatly and remained so, until 12th week.

The secondary response sera samples obtained from experimental animals and patients were separated into three well defined peaks on DEAE-cellulose at different salt gradients. Peak I, II and III corresponded to IgG, IgA and IgM, respectively. The immunoglobulins were separated on the basis of ionic charges present on the molecule in 0.0175, 0.035 and 0.15 molar phosphate buffer solution, respectively. Fractionation of antiamebic sera by Sephadex G-200 also resolved into three peaks. Peak I and II corresponded to IgM and IgG, while peak III contained a mixture of IgA and IgG along with albumin. Immunoglobulin A did not resolve separately on Sephadex G-200. The isolated immunoglobulins were then characterized against appropriate monospecific anti-immunoglobulin sera by using various antigen-antibody reactions.

The fractions obtained from DEAE-cellulose were further checked on Sephadex G-200 columns for heterogeneity. On the basis of their molecular size the isolated immunoglobulins were not found homogeneous. The isolated fractions from Sephadex G-200 seemed to possess some

heterogeneity, as evident from the results obtained in polyacrylamide gel electrophoresis.

The sequential sera samples obtained during primary and secondary responses were also similarly separated on Sephadex G-200. The immunoglobulin levels were also calculated from the pooled fractions. In the primary response sera the IgG and IgM were both present, but the hemagglutinin levels were quite low. Whereas in secondary response sera, the hemagglutinin and precipitin levels were found considerably increased. The level of IgM increased from 2 mg in 3rd week to 4.95 mg in the 5th week. The IgG level in the corresponding period increased from 13.77 mg to 14.95 mg.

The antiamoebic antibody activity detected by means of several tests revealed that major antibody activity was mostly confined to IgG. The test procedures used for this purpose included immunoelectrophoresis, immunodiffusion and fluorescent antibody tests. The results of IHA tests indicated that hemagglutinin activity was present in both IgG and IgM antibodies, while the precipitin activity resided only in IgG antibodies. During an active disease state, the level of IgG was always found elevated.

The isolated IgG and IgM fractions from the pooled antiamoebic serum showed 1:4096 and 1:1024 titre values as positive for hemagglutinin activity. Four precipitin bands were generally detectable against IgG, while only two bands appeared against IgM in immunodiffusion and immunoelectrophoresis tests, respectively.

The fractions from DEAE-cellulose were first rechromatographed on Sephadex G-200 and then used in various physico-chemical studies. The ultraviolet absorption maxima of IgG and IgA were found at 278 nm. A minor hump was detectable in IgG at 290 nm, indicating the presence of tyrosinase, tryptophan and phenylalanine. The absorption maxima of IgM ranging from 250 - 280 nm suggested that IgM contained more phenylalanine than IgG and IgA. The fluorescence spectral studies also confirmed the presence of tyrosine, tryptophan and phenylalanine in the three immunoglobulins.

Molecular weight of antiamobic IgG was calculated on the basis of gel filtration behavior, which was found to be 150,000. The Stoke's radius, frictional ratio and diffusion coefficient of anti-amebic IgG, as calculated on the basis of its gel filtration behavior data, was found as 4.613, 1.138 and 4.827×10^{-7} cm^2/sec, respectively. From these values it can be concluded that antiamebic IgG is a globular, but an extended molecule with an ellipsoidal or cylindrical shape.

Carbohydrate estimations were also carried out in chromatographically isolated immunoglobulins from antiamebic sera. The total hexose content of IgG, IgM and IgA were 1.06, 5.25 and 4.70 g/100 gram protein, respectively. Similarly, the N-acetylglucosamine content of the three immunoglobulins were 0.79, 0.96 and 0.32 g/100 gram protein. The sialic acid content of the three immunoglobulins were found as 2.5%, 2.34 and 0.53 g/100 gram protein, respectively.

The cell-mediated immune responses as obtainable against amoba antigen were demonstrated in immunized animals by making use of delayed

hypersensitivity reactions. Sensitised guinea pigs were found to give rise to a well defined intradermal reaction, when 8.8 ug and 4.4 ug of ameba antigen concentrations were used as intradermal challenging doses. The delayed hypersensitivity reactions were detectable two weeks after the primary immunization. A positive reaction was characterised by the appearance of erythema and induration after 24 hrs, and subsiding thereafter. Histological examinations were also carried out in order to study the types of cells present at the lesion sites. The lesion sites were characterised by the presence of a perivascular cuffing and a diffused infiltration of the intracellular spaces by lymphocytes and macrophages. A few polymorphonuclear leukocytes were also seen.

The appearance of CMI against ameba antigens in sensitized animals was further studied by carrying out an in vitro study of migration inhibition. The migration inhibition of peritoneal exudate cells was carried out in capillary tubes and on agarose plates. In capillary tube test, it was found that 0.88 ug antigen is the optimal antigen for studying the migration inhibition. The groups of animals receiving 70.5 ug of antigen during immunisation appeared to give a better immunological response. The migration inhibition of macrophages in the above immunised animal group (obtained by using 0.88 ug antigen during test) was 48.92 per cent. While the migration inhibition of macrophages in the animal group receiving 141.0 ug of antigen during immunisation (obtained by using 0.88 ug of antigen during test) was 9.8 per cent on agarose plates. The results in the two tests varied due to the differences in the employed technics. Amebic antigen preparations used in these two tests were crude and particulate type. These

extracts were, infact more appropriate for use in capillary tube tests. The values obtained in capillary tube test were slightly higher than those obtained by the average method.

CHAPTER - I

INTRODUCTION

A. HISTORICAL BACKGROUND

Entamoeba histolytica is a parasite of class Sarcodina, possessing pseudopodia, an organ responsible for locomotion and also for procuring food. The parasite, *E. histolytica*, is the causative agent for a variety of human syndromes or diseases, commonly referred to as amoebiasis. It is a cosmopolitan parasite encountered in almost all the regions of the world. The epidemiologic factors, infact, result in a higher incidence of infection in tropical and subtropical areas, affecting about 10 per cent of world's total population. The prevalence and severity of the infection may differ from area to area, or in a particular area it may also increase in special circumstances.

Amoebic dysentery is one of the oldest known diseases to mankind. The dysenteric illness described at Hippocratic time also included amoebiasis. Until the last quarter of the nineteenth century, infact, no distinction was known to exist between amoebic and other forms of dysentery. Lambl (1) in Prague in the year 1859 described the presence of living amoebae in the feces of a child, who had died with enterocolitis, pneumonia, anemia and hydrocephalus. Similarly, Losch (2) in 1875 had also observed the motile amoebae with ingested red blood cells in the feces of a patient suffering from dysentery. He also tried to prove the pathogenic nature of the amoebae by administering the contaminated fecal material orally and rectally to experimental dogs. The experimental dogs later developed

dysentery and, its feces contained the amebae. The autopsy also revealed the ulceration of the large bowel. Walker and Sellards (3) for the first time in 1913 succeeded in producing the disease in human volunteers. They also discovered the 'carrier state' in cases of non-invasive human amebiasis. The relationship of enteric bacteria to amebic virulence has more recently been investigated by Phillips et al. (4) and Taylor (5). Boeck and Drbohlav (6) in 1925 for the first time reported the successful cultivation of amebae in vitro, which they used for studying the disease in detail. Such cultures were always grown in association with one or several concomitant bacteria. But ameba-bacteria cultures were not very useful for making precise studies of the various physiological and immunological processes during the course of an infection, or otherwise. Soon it was realised that pure cultures of ameba must be employed for making more meaningful studies of the various mechanisms involved in infection and immunity. Cleaveland and Saunders (7) were the first who successfully transferred sterile ameba cysts from liver abscesses in cats to a sterile culture medium. But they could not maintain these cultures for very long. After few days, the amebae were all dead. Ukleny et al. (8) and Synder and Ukleny (9) tried to employ chemical treatment as a means of sterilizing the stool isolated cysts for in vitro ameba cultures. They also could not maintain their ameba cultures for more than two or three transfers. Rees (10) and Rees et al. (11) adopted a new technic of microisolation of cysts for initiating a pure culture. Antibiotics, were also subsequently used for eliminating the

accompanying bacteria from the cultures. Jacobs (12) for the first time demonstrated the usefulness of antibiotics in eliminating bacteria from amoba cultures. In 1948 Shaffer and Frye (13) successfully employed antibiotics for eliminating the associated bacteria from their cultures. Subsequently, Reeves et al. (14) substituted radiation inactivated bacterial cells for antibiotic-inhibited bacteria in amoba cultures. The growth of amoba with a single bacterial associate is generally referred to as a 'monobacterial culture', while the amoba cultures without any concomitant bacterial growth is known as an 'axenic culture'.

Stoll (15) for the first time tried to axenize the amoba cultures and succeeded in the cultivation of E. invadens in a cell free medium. In 1961, Diamond (16) has done remarkable work in axenizing E. histolytica cultures in vitro. He micro-isolated amebic cysts from amoba-bacteria cultures and later established them in an amoba-crithidia monoxenic culture. He was using a monophasic medium which was presoaked with a trypanosomatid of the genus Crithidia. After the establishment of monoxenic cultures, the amoba along with their flagellate associates were transferred to a specially devised diphasic medium. In this medium, the crithedia died out within three transfers and the amebae continued to differentiate and flourish in the form of pure or axenic cultures. In 1965 Diamond and Bartgis (17) further simplified their earlier culture technic by developing a monophasic liquid medium (18). The liquid

medium TP-S-1-monophasic (Tryptose-Penrode-Serum) is now mostly used for the cultivation of axenic cultures of E. histolytica. Such axenic cultures are free from contaminating organisms and their bye-products as well. These cultures can also be readily employed for growing the amebae en masse. The development of pure culture technics opened up new possibilities for making pure antigenic extracts.

Presently, seven species of parasitic amebae are known. These are: Entamoeba histolytica; Entamoeba hartmanni; Entamoeba coli; Endolimax nana; Entamoeba gingivalis; Dientamoeba fragilis and Jodamoeba butschlii. Out of these seven species, only E. histolytica is recognized as a true human pathogen and is now universally accepted as the causative agent of a typical human syndrome known as 'amebic colitis'.

These free-living amoeba have also been reported as human parasites, for example Acanthamoeba, Hartmannella, and Naegleria. In 1965 Acanthamoeba infection was reported as causing fatal meningitis in children and adults. The chronic granulomatous brain lesions have been produced experimentally in mice by the isolated Hartmannella Sp. from the soil. These isolates were found to have a relatively low virulence.

The life cycle of E. histolytica is completed essentially in two stages, the trophozoites (feeding stage) and the cystic (dormant) stage. The two stages alternate with each other.

Trophozoite grows at the expense of the host and multiplies into two equal halves by a process of binary fission. Cysts are not generally formed in the host tissues. In unfavourable conditions, some of the trophozoites in the intestinal lumen periodically eliminate their food particles and round themselves off to form a stage known as precyst. The vegetative functions generally cease at this stage and the precyst forms secrete a substance to form an enveloping membrane around the organism. That is how an amoeba cyst is formed. The cyst is quadrinucleate and represents a stage primarily meant for transmission only. The quadrinucleate cystic amoeba is the only stage which is transmitted from host to host. Their elimination from the feces and entrance into a new host through oral contamination is accomplished only by the cystic stages (19,20). During excystation after reaching a new host, the quadrinucleate cysts undergo repeated divisions until eight small trophozoites are formed from a single cyst in the host's intestine. In the case of avirulent strains, these organisms adopt a commensal existence in the lumen of the large intestine, feeding on bacteria and edible detritus.

B. PATHOGENICITY

There have always been some controversies among parasitologists regarding the pathogenicity of E. histolytica. These doubts were mostly based on the observations that a large number of infected hosts appear to be asymptomatic carriers only.

It was also believed that E. histolytica comprises of a group of similarly appearing amebae, and out of them only one strain has the pathogenic capability. On this score two schools of thought still exist, each one in direct opposition to the other. It is axiomatic that neither of the opposing view can be conclusively supported by experimental evidences. Craig (21) in his excellent treatise 'Amebiasis and Amebic Dysentery' stated that E. histolytica is essentially a tissue parasite which can not live as a commensal in the intestine, without producing lesions. There have been numerous other strict adherents to this theory including Calkins (22) and Dobell (23). On the other hand, several other workers including Elmassian (24), Reichenow (25-27) and Moore (28,29) believed that amba may only be living as a commensal in the intestine of its host, invading the tissue in comparatively rare instances. The principal evidence cited by Craig (21) in support of this theory was the work of Councilman and La Fleur (30), Bartlett (31), Kessel (32), Hegner et al. (33) and Craig and Kagy (34). All these workers have observed lesions which they attributed to E. histolytica in postmortem examinations of humans and experimental animals. The infected subjects, according to their reports, had no history of amebic dysentery during their life time. Similar observations were later reported by Faust (35). The arguments of the opposing groups are, infact, equally impressive. Reichenow (25) suggested that the number of amebae passed in the stools of carriers is greater than might be expected from small lesions. He further elaborated the fact that presence of amebae in carrier cases provide an indisputable proof

of their existence as commensals within the lumen. He also maintained that such amoebae when passed in feces contain ingested bacteria and other contents of the lumen with no evidence of ingested blood or other tissue cells. Andrews and Atchley (36) have reported that no traces of occult blood were found in the stools of a group of symptomless carriers of E. histolytica, citing this as evidence for the existence of the organisms as nonpathogenic commensals.

Bloody and Frye (37) appear to be of the view that pathogenicity amongst the various strains of E. histolytica manifests itself only in various grades or degrees. Alexander and Meleney (38), Bladen-Low (39), and Felsenfeld and Comess (40) have provided evidences to show that nutritional status of the host is an important aspect to consider before describing the pathogenicity of the organism. Deschiens (41,42), Chang (43), Lutterwood and Phillips (44) and Phillips and Bartgis (45) have shown that the pathogenicity of the organism may be altered by prolonged in vitro cultivation with microbial associates. Westphal (46) and Phillips et al. (47) have demonstrated that the amebae in some way are dependent upon their associated microorganisms for their pathogenicity. They believed that every strain of E. histolytica is capable of producing ulcerative changes in infected individuals.

The extensive ulceration, which characterises the disease in conventional host, is not observed in experimentally infected

germ free animals. The lesions produced in germ free animals are of a minor nature. As a nodular appearance, it generally remains confined to the exterior of the cecal wall. The amebae in germ free animals survive only briefly because of a very poor rate of multiplication in the lumen of such animals. As far as pathogenicity is concerned, it is not too well known whether the dependence of ameba on bacteria for its pathogenicity involves multiple bacterial species or only one. Pathogenic amebae in association with Escherichia coli, Aerobacter aerogenes and Bacillus subtilis have been amply shown to produce more severe ulcerations in experimental animals. On the other hand, the pathogenicity was found to decrease in in vitro association with Trypanosoma cruzi. Phillips et al. (47) have described the results of their studies on the pathogenicity of axenically grown amebae. They have found that the axenic amebae fail to produce lesions in germ free guinea pigs. They attribute this phenomenon to a loss of virulence by the ameba due to prolonged in vitro cultivation. The mechanism of the accompanying physiological changes from virulence to avirulence during its maintenance in axenic cultures is not exactly known. Similarly, no rational explanation can be put forward for the restoration of virulence after their continued serial animal passages (48-50).

C. MECHANISM OF PENETRATION

There are several theories relating to the mechanisms of pathogenesis in amebic infections. But these facts are not

experimentally proved. Ratcliff (51) believed that E. histolytica enters the tissue of its host by mechanical penetration. This was considered as the primary mechanism for the pathogenesis of the amebae. Later, Kenyon (52), Werner et al. (33), Councilman and La Flour (30), Craig (21) and Faust and Kagy (53) were of the opinion that mechanical penetration may only be just one of the factors involved. The other possible mechanism suggested by them is that the amoba may enter the tissue by cytolysis. The evidence for the existence of such an enzyme is based on histological studies of the lytic necrosis in the tissues. The trophozoites can be readily observed in the necrosed tissue which usually appears as a clear zone of lysis surrounded by healthy adjacent tissue. From these observations, it can be concluded that enzymatic ability along with the mechanical action are the two most likely factors which help the amebae in penetration and establishing the infection foci (53,54). Takeuchi and Phillips (55) and Pittman et al. (56) have shown in their electron micrographs a complete sequence of tissue invasion in amebiasis. The early changes in mucosa following an invasive infection include superficial ulceration in the form of a typical bottle-neck ulcers, followed by the destruction of the submucosa. The tissue damage is helped by proteolytic enzyme activity of the amebae. The presence of such proteolytic activity has been shown in in vitro enzymic studies. This is confirmed by the findings that microorganisms and their extracts both have the activity to hydrolyze the gelatin, casein, fibrin, hemoglobin, and guinea pig gut epithelium (57). Both pathogenic and nonpathogenic strains of E. histolytica have tryptic

and peptic activity, but this does not include the chymotryptic activity. The digestion of the gut epithelium by pepsin only is quite unlikely, because pepsin works within a pH range in which the parasites are usually inactive *in vitro*. The decline in the proteolytic activity is generally taken as an index of decreased pathogenicity. Although Neal (58) has recently stated that there exists no correlation between the increased proteolytic activity and a greater invasiveness. After invading the submucosal layer of the gut wall, the spread of the amebae into deeper tissues or mucosal region is assisted by the hyaluronidase enzyme. The above worker has shown the presence of enzymic activity in all the pathogenic organisms which were capable of producing average grade lesions in guinea pigs. Similar activity was also found in strains isolated from asymptomatic subjects. But any direct relationship between pathogenicity and nonpathogenicity can not be established on the basis of presence or absence of this enzyme only. It is also found that trophozoites from pathogenic and nonpathogenic strains of amebae have cytotoxic effect on leukocytes. This cytotoxic effect was mostly found on cytoplasmic granules and lysosomes which were completely disrupted. But a greater insight into pathogenic mechanisms of ameba can perhaps be obtained only after understanding the host-parasite relationship in its totality.

D. IMMUNE RESPONSES

The actual mechanism of generating immune responses against *E. histolytica* infections is not yet so well defined as in other

infections. During amebic infection, it is found that the generated immune response is comparatively weaker than the one obtained in similar type of infections by bacteria and viruses. The fact that an immunologically competent host remains fully susceptible to reinfection, even after a recent contact with the causative agent, is rather hard to define. Lack of information regarding the pathogenic mechanisms and, the exact nature and composition of antigens participating in the infection processes, are possibly some of the factors responsible for a poor understanding of the immune responses. At times, even the pathogenicity of the causal agent is somewhat doubtful. It has often been shown that the parasites within the lumen of the host exist only in a state of symbiotic relationship, without causing any apparent tissue damage (4). Similarly, the immunological sequence in such cases where extensive tissue damage is caused also remains equally obscure. Some of the more fundamental aspects of amebic immunology like innate, acquired, and passive immunity, nature of antigen and antibodies and humoral and cellular immune responses have now been studied in greater detail, though, by various workers.

a) Innate Immunity

Available evidences indicate that mammalian amebiasis is anthroponosis, i.e. the disease under natural conditions is restricted to humans only. Meare (59) and Dobell (20) have described the detection of similar types of amebae from monkeys in which the parasites run a symptomless course of infection.

Infact, there is not a single report clearly supporting the view, that a well established immunity against E. histolytica can be acquired as a result of natural infection or subsequent to immunization. Only Rogers (60) has reported spontaneous recovery from amoebic liver abscess and pleuropulmonary amoebiasis in a number of patients. These findings show that human body may have some mechanism of resistance against the invading amoebae. The results of Hegreith and Karimasuta (61) on experimental production of amoebic lesions in the liver of hamsters and guinea pigs have indicated that some type of natural immunity must exist in amoebiasis. They have found that amoebae were able to survive and grow in the hamster liver after being injected directly, or following secondary migration from existing intestinal lesions. But in the liver of guinea pigs, the amoebae failed to establish themselves. These findings confirm the view that a certain degree of resistance must be present in guinea pigs, but not in hamsters. On the basis of these finding it can not however be conclusively established that an innate immunity against amoebiasis is present in guinea pigs.

b) Acquired Immunity

Similarly, nothing is known about the acquired immunity against E. histolytica in man (62). There is no evidence to show the development of any resistance to reinfection, irrespective of a past history of recovery. The host is always susceptible to infection. In one investigation, it has been shown that some degree of protection was achieved in young rats after immunisation with

E. histolytica antigen (63). Wartzwelder and Avant (64) have shown the presence of acquired immunity in dogs. Such an immunity was demonstrated by assessing the degree of resistance to reinfection against E. histolytica. They have reported that the establishment of infection in nonimmune dogs was as high as 85 per cent. While in previously infected dogs, the infection rate was only about 12 per cent. The resistance to reinfection was equally effective against both homologous and heterologous strains of E. histolytica. The duration of reinfection ranged between two and a half months to nine and a half months. They have also detected that the rate of infection was much less in animals which received reinoculation blood transfusions from dogs refractory to infection. Compared to this, those animals which were not transfused were found highly susceptible. The above findings suggest that a passive transfer of immunity against amebiasis in dogs is possible. Later, Tanimoto-Weki et al. (65) and Vazaques-Chavedra et al. (66) have demonstrated that healed amebic infections in hamsters protect the animals from recurrent infections. More recently, Sepulveda et al. (67) have also shown the protection of animals against infection by axenic and monoxenic amebae. Krupp (68), who separated the antigen in four fractions on the basis of their size, has demonstrated a certain degree of protection in guinea pigs immunized with fractionated E. histolytica antigen. In the above investigation, groups of animals were separately immunized with isolated fractions and whole antigen preparation as well. The experimental animals were later challenged intracecally with monoxenic E. histolytica trophozoites. The animals were subsequently sacrificed for the

detection of cecal lesions. It was found that the animals immunised with high molecular weight antigen fractions were completely protected. Whereas, only 43 and 64 per cent protection was achieved in animals immunized with other two fractions. Animals immunized with whole antigen showed protection in about 70 per cent cases.

The presence of innate, acquired and passive immunity has been variously shown in animal models. But such type of studies are altogether lacking in human subjects. Some studies have also shown that antiamebic antibodies are capable of exerting a cytopathic action on E. histolytica trophozoites in the presence of complement. Such antibodies were shown to be protective against reinfection. The metal ions (like Ca^{++} and Mg^{++}) enhance the cytopathic action, resulting in a progressive destruction of the membrane and disintegration of the parasites. Ahmad and Bisati (69) have also demonstrated in axenic cultures the presence of cytopathogenic effect of human anti-amebic immune serum and gamma globulin on E. histolytica (VIII: 200). Heat inactivated 30 per cent antiamebic human serum including gamma globulin, lost its growth inhibiting property. Addition of guinea pig complement resulted in a marked inhibitory effect. It was also demonstrated that human immune serum was able to neutralise the virulence of E. histolytica (70).

c) Nature of Antigen

Characterisation of ameba antigens, prior to the develop-

ment of pure culture technics, almost always yielded uninterpretable results. Diamond (16) has developed the axenic culture technics for cultivation of amebae in pure culture and for obtaining a pure antigen, as well. A large number of workers (71-76) have characterized the amebic antigens on the basis of their immunogenic behavior. Such studies have been carried out by studying antigen-antibody reactions. These workers have also detected 1 - 10 precipitin bands in E. histolytica antigen-antibody system by using gel diffusion technics. Recently, Krupp (77) evaluated 11 amebic antigenic components by immunoelectrophoresis. The lines observed in an agar-gel precipitin test represent the minimum number of antigenic components that are at equivalence with the anticmebic serum. Similarly, the antigenicity of various other strains of Entamoeba has also been studied by gel diffusion technics (78). A set of precipitin lines generally reflect the structure of a number of constituent proteins which are the direct products of the genome of a species. Different methods have been used for the fractionation of antigen. These are: sephadex column chromatography, cellulose ion exchange, electrophoretic mobility differences and density gradient centrifugation. Using these technics, Lewis and Kessel (79) did a pioneering study on fractionation of ameba antigens. They fractionated the monoxenic antigen extract on Sephadex G-200. The antigen extract was shown to resolve into five different peaks. The studies on molecular weights ranged between 180,000; 80,000; 25,000; 10,000 and 1,000 corresponding to their resolutions in peak I, II, III, IV and V respectively. All the fractions showed antigenic activity in various antigen-antibody

tests. Ali Khan and Meerovitch (30,31) have fractionated the aqueous extract of DK3 strain. Antigen extract from monoxenically cultivated amebae was separated into seven fractions in Sephadex G-200 columns. They also calculated the molecular weights of various fractions, which ranged between 1450 to 650,000. Most of the antiamebic activity was found confined to two high molecular weight fractions of 650,000 and 229,000 (32). These antigens were glycoprotein in nature, having both acid and basic proteins. Polyacrylamide gel electrophoresis studies showed the charge heterogeneity in various fractions obtained from gel filtration. These proteins were found as potent antigens.

In spite of these studies, further work is needed to understand the physical, chemical and immunological homogeneity of the antigens. Only then further insight can be gained for identifying the functional antigens and their specificities.

d) Nature of Antibodies

The appearance of immunity against several diseases caused due to parasitic infections is attributed to the formation of specific serum proteins known as 'antibodies'. The antibodies are produced by vertebrates in response to foreign invaders known as 'antigens'. Antibodies are also presently termed as immunoglobulins. On the basis of their structural antigenic determinants, the immunoglobulins are divided into five major groups or classes: IgG, IgM, IgA, IgD and IgE. Smithers (83) has shown that in various parasitic

diseases, an elevated level of immunoglobulins is present. Such antibodies have been detected by a large number of workers by using antigen-antibody reactions (84). Anderson et al. (85) have reported the elevated levels of α-2-globulins and γ-globulins in acute intestinal amebiasis. A large number of serological reactions are available for the detection of serum antibodies for the diagnosis of the disease. But very little is known about the nature of such antibodies which participate in these antigen-antibody reactions. Not much is either known about the dynamics of antibody response, which must actually depend upon the nature of antigen and the antigenic stimulus. Maddison et al. (86) have characterized the antibodies formed in response to E. histolytica infection in man. Immunoglobulins (IgG, IgA and IgM) were separated from the patient's sera on cellulose and Sephadex G-200 columns. Ali Khan and Berovitch (34) have fractionated the primary and secondary response sera samples. These sera were obtained at weekly intervals from immunized rabbits and were fractionated on Sephadex G-200 columns. The above workers have reported simultaneous appearance of IgM and IgG antibodies during the primary response. The hemagglutinin activity was found in both the IgG and IgM fractions, while the precipitin activity was found mainly confined to the IgG fraction. In the secondary response, the IgG antibody increased greatly and persisted up to 12th week or longer upto a maximum of 6 months. The IgM level was also found increased. But this was only transitory - the reactivity was found to decrease very soon. Maddison et al. (87) have also shown the presence of skin hypersensitivity of the immediate type in clinical amebiasis. They have

also demonstrated that amebiasis immune sera, on being transferred to guinea pigs, was also responsible for giving rise to passive cutaneous anaphylaxis (PCA) reaction in sensitized animals. Later workers have also demonstrated in guinea pigs the presence of IgE showing a positive PCA reaction. Immunoglobulin A has not been characterized in amebiasis. Although it has been detected in the colon of other types of infection (88,89). It is reported by Lap et al. (90) that immobilization factors against E. histolytica was present only in IgG fraction which was isolated on DEAE-cellulose and Sephadex G-200 columns. Later, Abioye et al. (91) have demonstrated that IgG is the main reactive antibody in invasive amebiasis. They have also estimated the level of IgG in different kinds of patients. On the basis of these studies, it was concluded that a relationship must exist between IgG levels and invasive amebiasis. Several other workers (92,93) have also reported that indirect haemagglutination and electrophoresis tests gave positive results in 90 per cent patients having liver abscesses. The antibody activity was largely confined to IgG fraction, and that such activity was specific for E. histolytica antigen only.

E. HUMORAL IMMUNE RESPONSES

After an infection, two types of immunological responses are generated - the 'humoral' and 'cell-mediated'. In humoral responses, there is a synthesis and release of circulating antibodies into the blood stream. These antibodies act directly on the foreign intruders and neutralise the infection by coating themselves

on the causative agent, and also by enhacing the rate of phagocytosis. In cell-mediated immune responses, the 'sensitized' lymphocytes having antibody-like molecules on their surfaces (cell-bound antibodies) are produced. These are the effectors of cell-mediated immunity. Their activity is generally expressed in such reactions as the rejection of skin transplants and 'delayed' type of hypersensitivity to the specific antigens or infections.

When B lymphocytes are activated by antigen, they divide and differentiate into blast cells, developing finally into plasma cells. These plasma cells are capable of actively synthesizing and secreting specific immunoglobulin antibodies. The plasma cells have a well developed rough surfaced endoplasmic reticulum which is characteristic of a cell producing protein for 'export'. As for humoral immune responses, it is commonly seen that during an amebic infection high level of antibodies are detectable by different serological tests. These tests are widely employed for diagnostic as well as for epidemiological survey work. Presently, precipitin test (74,94-102), indirect hemagglutination (IHA) test (95,103-111), complement fixation (CF) test (112-116), ameba immobilization test (90,109), fluorescent antibody (FA) test (117-120), latex agglutination (121-123) and bentonite slide flocculation (BSF) tests (124) are some of the commonly employed methods for the detection of antiamebic antibodies. From these tests, it has been found that specific antiamebic antibodies are chiefly localized in IgG and IgM fractions (86,90,91,93). Scales (125) and Leal (126) have reported immediate type hypersensitivity reaction in amebiasis patients.

Later, Heathman (127) and Menendez (128) have also demonstrated a delayed type of hypersensitivity against E. histolytica in hyperimmunised animals. Ishizaka and Ishizaka (129) have shown that immediate type of hypersensitivity is due to reaginic immunoglobulin E. Sera samples from patients, showing a positive immediate type hypersensitivity reaction, were also tested for the appearance of passive cutaneous anphylaxis (PCA) in guinea pigs and monkeys (76,130).

All the above tests amply demonstrate the presence of precipitin and hemolytic activity in antiamebic antibodies. Such activities are mostly confined to IgG and IgM antibodies. But the IgA antibodies are devoid of any such activity. It was also demonstrated that IgE antibodies are responsible for giving a positive PCA test. During an amebic infection, it is commonly observed that the level of humoral antibodies is raised. Although the patient is not protected against E. histolytica infections. In the beginning of the disease, only immediate type of hypersensitivity reactions can be demonstrated. Whereas, the delayed type of skin hypersensitivity reactions can only be demonstrated in the cured patients of liver abscesses. From these studies, it may well be concluded that circulating humoral antibodies are abundantly produced in amebic infections. But these antibodies are not of a protective nature. Such antibodies do not afford any protection to the host, although their level in the blood remains significantly raised. The only evidence which supports the view that some protection is possible in amebic liver abscesses is perhaps provided

by the appearance of a delayed hypersensitivity reaction (CMI) in such cases.

F. CELL-MEDIATED IMMUNE RESPONSES

The effector cells of cell-mediated immunity are T lymphocytes. These thymus-dependent lymphocytes arise from stem cells in the bone marrow and mature under the influence of thymus. They are unable to produce antibodies as such, but are responsible, at the same time, for mediating cellular immunity. When stimulated by specific antigen, these T lymphocytes liberate several soluble substances or factors known as transfer factor (TF) and migration inhibitory factor (MIF). In general, all these factors are collectively known as lymphokines (131,132). These lymphokines are, infact, the mediators of cell-mediated immunity. The transfer factors in a sensitized host assume an important function by converting some of the nonsensitive lymphocytes to an antigen-responsive state. Such a recruitment phenomenon would be important, since only very few of the lymphocytes are initially sensitive to the specific antigen. Once produced, the lymphokines do not loose their activity even after the removal of the antigen from the culture, or system. Lymphokines are also released by lymphocytes, which are activated by nonspecific mitogens such as phytohemagglutinin and concanavalin A (133,134) in antigen-antibody complexes (135) and mixed lymphocyte cultures (136). In guinea pigs, antigen induced macrophages not only produce MIF but also inhibit the migration of exudate cells. Purified lymphocytes or macrophages

from sensitised animals are not inhibited by the presence of antigen alone (137,138). The cell purified population is inhibited only by antigen when purified lymphocytes are added to the isolated macrophages, or vice-versa (137). The lymphocytes are immunologically active cells, whereas macrophages only act as indicator cells capable of migration.

Delayed hypersensitivity (DH) reaction is almost always associated with cell-mediated immunity (CMI). Three different types of skin reactions have been reported in amebiasis. These are immediate type, Arthus type and delayed type skin hypersensitivity reaction. The immediate type of skin reaction is associated with the reaginic type of IgE antibody (129). This type of reaction develops just after injection of antigen and reaches to its maximum in about 15 - 30 min. Such a reaction is characterised by the appearance of a wheal and flare reaction and fades out in 1 - 2 hrs. Mostly, the IgE antibodies remain bound to the mast cells. When they come in contact with antigen they liberate certain pharmacologically active substances such as histamine, serotinin and bradykinin. These substances are responsible for immediate type of hypersensitivity reaction. The Arthus type of reaction is mediated by IgG antibodies when it gets complexed with antigen and complement. In this reaction, the patients develop an erythematous and oedematous reaction, reaching a peak in 3 - 8 hrs. The delayed hypersensitivity reaction is characterised by erythema and induration which appears only after several hrs (12 hrs), reaching a maximum at 24 - 48 hrs and subsiding thereafter. Histologically, these skin reactions are

characterised by the type of cell population present at the reaction site. In immediate type of hypersensitivity reaction, the eosinophils are the main predominant cell population, while in an Arthus reaction the polymorphonuclear (PMN) neutrophils are the main cell types. In delayed types, mostly lymphocytes and the cells of monocyte-macrophage series are present.

Heathman (127) and Fernandes (128) in 1932 for the first time demonstrated the delayed type of hypersensitivity to E. histolytica in experimentally immunized animals. Heathman (127) was also able to transfer (passively) skin-sensitizing antibodies in recipient animals from the immunized ones. Maddison et al. (87) have also employed the skin reaction for correlating its activity with IHA and FA tests. These tests were carried out in the patients of active invasive amebiasis and in a comparable asymptomatic group by using monoxenically prepared DKB antigen. They have reported that patients of invasive amebiasis showed 81 per cent positive skin reactions. Whereas, 100 per cent cases were found positive in agar gel and IHA tests. A ninty per cent positive results were obtained in FA test. In asymptomatic group, only 14 per cent were found positive in agar-gel, 32 per cent in IHA and 9 per cent in FA tests. These authors reported that dermal reactions from active disease states predominantly belonged to the immediate type. In asymptomatic group of patients, the obtained reactions were of the delayed type. Kretschmar et al. (139,140) used axenically cultivated E. histolytica antigen (HK-9 strain) for studying intradermal reactions in liver abscess patients and, also in others showing

positive symptoms of invasive amebiasis. After giving an intradermal injection of antigen to the patients, the appearance of skin reactions were observed at 5, 24 and 48 hr intervals. They found that 57 per cent gave a positive skin reaction in invasive amebiasis, 47 per cent in intestinal amebiasis and 20 per cent in cases of asymptomatic patients. They also reported using the various antigen fractions (isolated on Sephadex G-200) in their intradermal tests given to patients of amebic liver abscesses and to healthy normal controls. These studies have confirmed that delayed type of hypersensitivity reactions are found only in active patients, but not in normal controls. The first two fractions (I and II) possessing high molecular weights were found to give potent and clearly defined skin reactions, even when used in lower doses (4 ug/ml). Similar type of skin reactions were also demonstrated in amebic patients (141). Landa et al. (142) have also shown the presence of delayed type of hypersensitivity in patients suffering from acute amebic liver abscesses. The sera from these patients were also found positive for humoral antibodies as detectable by counterimmunoelectrophoresis. The patients were tested for the appearance of skin reactions by injecting 6 ug of axenic amebic antigen along with Streptokinase - streptodornase and PPD during the first week of the disease, and a month after healing. In the beginning, the rosette test for T lymphocytes was normal in 80 per cent cases. At the onset of the disease, the skin tests for amebiasis were positive in 16 per cent of cases. Whereas, those of streptokinase-streptodornase and PPD were positive in 68 per cent cases. While in the later stages of the disease, the skin tests were found positive in 64 per cent cases.

The streptokinase-streptodornase and PPD combination gave positive results in 84 per cent cases. These findings strongly suggest the involvement of cell-mediated immune responses in human amebic infections. Similar types of responses were also obtainable from guinea pigs by Lunde et al. (143).

Besides this, some other parameters were also used to assess the presence of cell-mediated immunity during amebic infections. Recently, several other workers (144-146) have also evaluated the appearance of CMI in patients and experimental animals in an in vitro system. These studies were made on the basis of an in vitro interaction between patient's leukocytes and ameba trophozoites. It was concluded from these studies that the lymphocytes obtained from patients, exerted a cytotoxic effect on pathogenic trophozoites. A similar effect was also observed when trophozoites were mixed with the lymphocyte supernatant obtained after 24 hrs stimulation of the lymphocytes with specific ameba antigen. On the basis of these studies, it was concluded that antigen-sensitized T lymphocytes release some cytotoxic factors which are capable of distorting the trophozoites. Such cytotoxic effect was mostly associated with cellular immune responses only (147).

Blast transformation of antigen sensitised lymphocytes is another in vitro method which has been used to study the CMI responses in amebiasis (148). These blast transformation studies have been reported for the first time in amebiasis. Peripheral blood lymphocytes from amebic liver abscess were stimulated in vitro

by an aqueous extract of E. histolytica. Blast transformation of lymphocytes was measured by their ability to incorporate tritium thymidine. Blast transformation of lymphocytes is a very specific process and is as such, transformed only by specific antigens. It has also been demonstrated that blast transformation was not correlated with the production of humoral antibodies, because the ability of blast transformation in the presence of specific antigen did not appear related to the number of precipitating bands in immunoelectrophoresis. Similar type of results have also been reported in the lymphocytes obtained from amebiasis patients (149).

The migration inhibition technic was also used to evaluate the CMI responses in amebic patients. Ortiz-Ortiz et al. (150) have reported that patients with amebic liver abscesses show a lower frequency rate to the appearance of delayed-type reaction. These patients were tested by using skin reactions and MIF production. The results were compared with similar reactions observed after 10 days. In the present studies, it has been found in guinea pigs that the antigen-sensitized macrophages are inhibited from migration by specific antigen. On the basis of these and other earlier cited investigations carried out in vivo and in vitro, it could be concluded that CMI responses are not only functional but also important in amebiasis.

AIMS AND OBJECTS

The main aim of the present investigations was to characterise the immune sera samples from cases of human invasive amebiasis and as well as from antigen sensitized animals. Studies were also undertaken to evaluate the cell-mediated immune responses in antigen-sensitized animals. A detailed study plan was carried out as follows:

1. Water soluble antigenic extracts were prepared from the laboratory cultivated axenic E. histolytica (Strain HH: 200).

2. Animals were sensitized with water soluble antigenic extracts for the evaluation of humoral and cell-mediated immune responses.

3. Antiamebic antibodies were detected in immune sera samples by using different antigen-antibody reactions.

4. Humoral antibody responses were also studied by detecting and evaluating antiamebic antibodies in primary and secondary response sera samples obtained from antigen-sensitized animals.

5. The appearance of immunoglobulins in primary and secondary immune sera samples was monitored by measuring the immunoglobulin levels. The immunoglobulin levels were measured by protein estimations in each fraction which was obtained from column chromatographic separations.

6. The immunoglobulins from normal and immunized animals were initially separated by making use of chromatographic separations on DEAE-cellulose and Sephadex G-200 columns. The purity of the isolated immunoglobulin fractions was checked by PAG electrophoresis.

7. The specific antibody activity of the isolated immunoglobulins was also detected by making use of several antigen-antibody reactions.

8. Isolated immunoglobulin fractions were used for physical and chemical studies such as fluorescence spectra, ultraviolet spectra and carbohydrates estimations.

9. Pure IgG fractions were used for studying the molecular weights, Stoke's radius, diffusion coefficient and frictional ratio for the assessment of size and shape of the molecule.

10. Animals sensitised from soluble antigen extracts were used to study the delayed hypersensitivity reactions by using skin tests. Histological studies of skin reaction sites were also carried out to identify the type of hypersensitivity reaction in the sensitised animals. These studies were also carried out to characterise the type of cells present at the reaction site.

11. The cell-mediated immune responses in guinea pigs, obtainable as a result of antigen sensitisation were further detected by studying an *in vitro* technic of macrophage migration inhibition in capillary tubes and on agarose plates.

CHAPTER - II

MATERIALS

1. Animals

Young, healthy guinea pigs of either sex weighing between 400 - 500 grams were supplied by Animal House, State Serum Institute, Copenhagen, Denmark. Antisera against E. histolytica antigen were raised in young male rabbits which were obtained from Animal House, Faculty of Medicine, Aligarh Muslim University, Aligarh.

2. Media

Tissue culture medium TC-199 was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. All the amino acids and vitamins used in the experiments were purchased from E. Merck, Germany. Trypticase was purchased from B.B.L., U.S.A. The Panmede was obtained from Pain and Bryne Limited, England. Calcium pantothenate and Pyridoxine hydrochloride were purchased from B.D.H., India; L-cystein hydrochloride, Para aminobenzoic acid and Tween-80 were obtained from Riedel, Germany.

3. Proteins

Crystalline ovalbumin, bovine serum albumin and α-chymotrypsinogen-A (type II, 6 x crystallised, salt free) were purchased from Sigma Chemical Company (U.S.A.). Gamma globulin (human) and Sheep IgG were obtained from Institute of Serobacteriology, Budapest, Hungary.

4. Sera

Horse and normal guinea pig sera were obtained from State Serum Institute, Copenhagen, Denmark.

5. Preservatives and Fixatives

Sodium azide was purchased from Riedel, Germany, Thiomersal from B.D.H., England and Glutaraldehyde from E. Merck, Germany. Heparin was purchased from Leo, Copenhagen, Denmark. The Penicillin and Streptomycin were obtained from Difco, U.S.A.

6. Gels

Sephadex G-200, Blue Dextran 2000, Dextran 250 and Ficoll were purchased from Pharmacia Fine Chemicals, Sweden. DEAE-cellulose, (Whatman DE-11) was purchased from W & R Balston Ltd., England. Triosil (Nyegaard & Co., Oslo-Norway), Ion agar and agarose were obtained from Difco, U.S.A. and Litex, Glostrup, Denmark, respectively. Bentonite was purchased from Evans Medical Ltd., Liverpool, England.

The chemicals used in polyacrylamide gel electrophoresis and their sources were as follows: acrylamide, (E. Merck, Germany); N,N,N',N'-tetramethylenediamine (Fluka, Switzerland), dichlorodimethyl silane (E. Merck, Germany), amidoschwarz 10 B (E. Merck); ammonium sulphate (Riedel, Germany) and bromophenol blue (B.D.H., England).

7. Antigen

Antigen samples of *Entamoeba histolytica* strain NIH: 200 were purchased from Wellcome Bourough Laboratories, England.

8. Buffers and Other Diluents

Analytical grade sodium carbonate, potassium chloride, calcium chloride, sodium phosphate (monobasic), sodium phosphate (dibasic), sodium bicarbonate, sodium chloride, sodium hydroxide, copper sulphate, sodium potassium tartrate, lithium sulphate, sodium tungstate, phosphoric acid, citric acid, sodium citrate, ammonium sulphate, sulphuric acid, hydrochloric acid, diphenylamine, perchloric acid, acetaldehyde, bromine and formalin were all purchased from B.D.H. (India). Tannic acid was purchased from W.J. Bush & Co. Ltd., England.

9. Sugars

Sugars like glucose, fructose, galactose and mannose were purchased from B.D.H., India. N-acetylglucosamine and sialic acid were purchased from Sigma and Company, U.S.A.

10. Dialysis Tubings

Dialyser tubings were purchased from Arthur H. Thomas Company, U.S.A.

All the chemicals used in the experiments were of analytical grade and were mostly obtained from commercial sources. All glass, double distilled water was regularly used in the experiments throughout these studies.

CHAPTER - III

METHODS

A. CULTIVATION OF AXENIC ENTAMOEBA HISTOLYTICA

Axenic cultivation of E. histolytica (Strain NIH: 200) was accomplished in TP-S-1-monophasic medium according to the method of Diamond (16,18). It is a liquid medium consisting of TP-nutrient broth, inactivated bovine serum and vitamin mixture No. 107. The medium was prepared as follows:

a. Preparation of Trypticase-Panmede (TP) Broth

A nutrient broth containing Trypticase, 1.0 g; Panmede, 2.0 g; Glucose, 0.5 g; Sodium chloride, 0.5 g; Ascorbic acid, 0.02 g; Potassium phosphate (dibasic), 0.10 g; Potassium phosphate (monobasic), 0.06 g and L-cystein monohydrochloride, 0.10 g, was prepared by dissolving the above reagents in double glass distilled water. The final volume was brought to 87.5 ml at a pH of 7.2 with 1N NaOH and filtered through one layer of Whatman filter paper No. 1. The preparation was dispensed in 43.75 ml volumes in 60 ml screw capped tubes and sterilized at 12 lbs pressure for 15 min. The broth stored at 4°C was normally used up within a period of one week.

b. Preparation of Vitamin Mixture No. 107

A vitamin mixture No. 107 originally developed by Evans and her associates (151) in 1956 and containing the following ingredients was prepared as per below:

1. Water Soluble B Vitamins

(a) Solution A: 62.5 mg niacin and 125 mg p-aminobenzoic acid were dissolved in boiling distilled water and brought to a total volume of 150 ml.

(b) Solution B: 62.5 mg niacinamide, 62.5 mg pyridoxine hydrochloride, 62.5 mg pyridoxal hydrochloride, 25 mg thiamine hydrochloride, 25 mg calcium pantothenate, 125 mg i-inositol and 1,250 mg choline chloride were dissolved in double distilled water and brought to a total volume of 150 ml.

(c) Solution C: 25 mg riboflavin was added to 75 ml of distilled water and dissolved with the aid of 0.1N sodium hydroxide by adding drop by drop. The total volume was then brought to 100 ml.

Solution A, B and C were then mixed together and the total volume was brought to 500 ml by the addition of distilled water.

2. Biotin Solution

30 mg D-biotin was dissolved in 200 ml distilled water with the aid of 0.1N NaOH and the total volume brought to 300 ml.

3. Folic Acid Solution

30 mg folic acid was dissolved in 200 ml distilled water with the help of 0.1N NaOH and the total volume brought to 300 ml.

4. Lipid-Soluble Vitamins A, D and K

(a) Solution A: 3.0 mg vitamin A_2 (calciferol) was dissolved in 63 ml of 95 per cent (V/V) ethyl alcohol. To 300 mg of vitamin A, appropriate volume of crystalline alcohol was added for dissolving.

(b) Solution B: 60 mg vitamin K (menadione sodium bisulphite) was dissolved in 300 ml of a 5 per cent (V/V) aqueous solution of Tween-80.

Solution B was then mixed with Solution A and the total volume brought to 3 liter with the addition of distilled water.

5. Vitamin E Solution

25 mg of vitamin E (alpha tocopherol acetate) was dissolved in 250 ml distilled water.

The working mixture of vitamins was prepared by mixing the five primary stock solutions in the following proportions:

1.	Water-soluble B Vitamins	-	500 ml
2.	Biotin solution	-	250 ml
3.	Folic acid solution	-	250 ml
4.	Lipid-soluble vitamins A, D & K	-	2,500 ml
5.	Vitamin E solution	-	250 ml

The clear vitamin mixture was then sterilized by filtration through a Seitz filter and stored at -20°C.

c. Bovine Serum

Fresh bovine blood was routinely obtained from the local slaughter house in a 250 ml conical flask and kept at room temperature till coagulation. After detaching the clot from the glass walls, the flask was kept in the refrigerator overnight. The clear serum was separated by centrifugation. Separated serum was inactivated at 56°C for 30 min in a waterbath. The inactivated serum was filtered, sterilized and stored in 60 ml screw capped tubes in a deep freeze at -20°C.

d. Preparation of TP-S-1-Monophasic Medium

To each tube containing 43.75 ml of sterilized Trypticase-Panmede broth was aseptically added 5 ml of sterilized inactivated bovine serum and 1.25 ml of vitamin mixture 107. All such medium tubes, before being used, were routinely incubated at 37°C for 24 hrs for checking the sterility of the medium.

e. Maintainance of Axenic Ameba Cell Line

Axenized NIH: 200 strain of E. histolytica, used in these studies, was isolated by John E. Lobie (152) in 1949 from Sigmoidoscopic material obtained from a patient with acute amebiasis. The axenization of this strain was accomplished by Diamond (16) in 1961. In this procedure, amebic cysts microisolated from ameba-bacteria cultures were introduced bacteria free into a specially prepared monophasic medium seeded with a trypanosomatid of the genus Crithadia. After establishment of these monoxenic cultures, the amebae along with their flagellate associates were transferred to a specially devised diphasic medium. In this culture, the Crithadia died out within three transfers, while the amebae continued to multiply and flourish in the axenic tubes.

For routine subculturing, a 96 hrs old culture was initially chilled in an ice water bath for about 10 min. This caused most of the amebae to fall off from the walls of the glass tubes. Each tube was also vigorously rotated between the palm of the hand to dislodge the remaining amebae. The ameba counts were regularly made in a Neubauer hemocytometer counting chamber. About 15,000 to 20,000 amebae/ml were generally used to inoculate a 12.5 ml portion of the medium. The inoculated tubes were incubated at 36.5°C. Subculturing at 72 and 96 hr intervals was a routine practice during the course of these investigations.

f. Mass Cultivation of Axenic Culture

12.5 ml volume of culture medium showing heavy growth of E. histolytica was asceptically dispensed in duck-shaped screw capped flask containing 50 ml TP-S-1-monophasic liquid medium. The culture flasks were incubated at 36.5°C for 96 hrs at the end of which the amebae were harvested. All ameba counts were made in the hemacytometer counting chamber.

B. PREPARATION OF WATER SOLUBLE WHOLE ANTIGEN

The 72 and 96 hrs old ameba cells were collected and washed three times in cold physiological saline. Cell packed volume of harvested amebae was obtained by centrifugation at 800 rpm for 10 min. The ameba cell packed volume was diluted to ten times by adding 10 ml of saline. The cell suspension before being subjected to disintegration was left in the refrigerator for 2 hrs. The cells were mechanically disrupted and lysed by passing them several times through a 20-g needle attached to a 20 ml syringe by the method of Kessel et al. (113). Subsequently, the cells were subjected to alternate freezing and thawing until all the amebae were completely lysed. This was confirmed by examining a drop of cell suspension in the microscope. The soluble antigenic material was separated by centrifugation at 20,000X G for 20 min. The merthiolated (1:10,000) antigen samples were dispensed in small vials and stored at -20°C.

C. DETERMINATION OF PROTEIN CONCENTRATION

All protein estimations were made according to the method of Lowry et al. (153) by using bovine serum albumin (BSA) as the standard. Folin-phenol reagent was prepared according to the method described by Folin and Ciocalteu (154). This solution was stored in brown colored bottles in the dark.

Copper reagent was prepared by mixing 2 per cent stock solution of sodium carbonate in 0.1N sodium hydroxide and sodium potassium tartrate along with 1 per cent of copper sulphate in the ratio of 100:1:1 (V/V/V).

To each 0.1 ml of protein sample, brought up to 1 ml by the addition of buffer or water, was added 5 ml of freshly prepared copper reagent. After incubation for about 10 min at room temperature, 1.0 ml of 1:4 diluted Folin-phenol reagent was added. The color intensity was read after 30 min at a wavelength of 700 nm. The protein concentrations were subsequently determined from bovine serum albumin standard curve.

D. pH MEASUREMENT

All pH measurements were made on an Elico model L_1 - 10 pH meter. Sodium tetraborate (0.1M, pH 9.18) and potassium hydrogen pthalate (0.05M, pH 4.0) were used as standard buffer solutions to calibrate the pH meter.

E. PREPARATION OF ALUMINA ADJUVANT

Aluminium chloride (20 g) was dissolved in 1.0 litre of distilled water. The solution was heated till boiling. After it cooled off, an excess of ammonia was added to it. The excess ammonia was later boiled off and the solution was centrifuged. The precipitate was suspended in distilled water and centrifuged again. The precipitate was further washed three times with distilled water. The precipitate was later transferred to a conical flask, suspended in water and heated till boiling. One ml aliquots of 0.1N HCl were gradually added with stirring till the suspension turned opalescent white.

The white suspension of alumina adjuvant was subsequently used for immunizing the experimental animals.

F. ANIMAL IMMUNIZATION

a. Immunization for Obtaining Humoral Immune Response

The antigen samples, which were first thoroughly emulsified in an equal volume of alumina adjuvant, were administered in rabbits by intraperitoneal and subcutaneous injections (78).

Male healthy adult rabbits were immunized with E. histolytica NIH: 200 strain whole-antigen preparation. The first injection which contained 0.5 ml of the above antigen was given intraperi-

toneally and also subcutaneously at different sites. Subsequently, the animals were inoculated with 1.0 ml antigen samples in the second and the third weeks. After a period of rest in the fourth week, a booster shot containing 2 ml antigen was finally administered. The blood was drawn at weekly intervals from the heart for checking the antibody activity and for detecting the sequential appearance of immunoglobulins in weekly sera samples. The immunization as well as the bleeding schedule followed during the course of these investigations is given in Table I.

b. Collection of Antiserum

After completing the entire course of immunization, small samples of blood were initially withdrawn for detecting the circulating antibody activity. Animals showing a good antibody response were used for obtaining blood directly from the heart.

I. HUMORAL ANTIBODY RESPONSES

A. DETECTION OF ANTIBODIES

The serum samples obtained from immunized rabbits were checked for the presence of specific anti-*E. histolytica* antibodies by the following tests:

1. Precipitin Ring Test

Precipitin titrations by the method of Maurer (155) were used

TABLE - I

Immunization and Bleeding Schedule in Rabbits.

Rabbits	WEEKS						
	I	II	III	IV	V	VI	VII
IMMUNIZATION							
1. Control							
Saline	0.5 ml	1.0 ml	1.0 ml	Rest	2.0 ml		
Adjuvant	0.5 ml	1.0 ml	1.0 ml		0.0 ml		
Total volume	1.0 ml	2.0 ml	2.0 ml		2.0 ml		
2. Experimental							
E. histolytica antigen	0.5 ml	1.0 ml	1.0 ml		2.0 ml		
Adjuvant	0.5 ml	1.0 ml	1.0 ml		0.0 ml		
Total volume	1.0 ml	2.0 ml	2.0 ml		2.0 ml		
BLEEDING							
1. Control	7th day	14th day	21st day	28th day	35th day	42nd day	49th day
2. Experimental	7th day	14th day	21st day	28th day	35th day	42nd day	49th day

Blood samples were also initially obtained from the control and experimental animals before immunization.

to detect t e positive antigen-antibody reactions and to determine the antibody titres as well in the immune serum. Thin walled precipitin tubes measuring 40 mm X 4 mm (internal diameter) were used for this purpose. One tenth milliliter of antiserum was introduced into each of the eight tubes in a series. An equal volume of serially diluted (1:10 through 1:10,000) antigen was carefully layered over the antiserum without mixing the contents, with the help of micropipettes. Normal rabbit serum, immune rabbit serum and saline controls were included in the tests. The tubes were incubated at room temperature for 4 hrs and then kept in the refrigerator overnight. The tubes were subsequently observed for the formation of precipitin rings at the interface.

2. Bentonite Slide Flocculation (BSF) Test

The bentonite slide flocculation test for the detection of serum antibodies was done according to the method of Bozicevich et al. (156), Norman et al. (157-159), Kagan et al. (160,161) and Tupasi and Healy (124). Serum samples from normal and immunized rabbits were inactivated at 56°C for 30 min. In performing the test, one drop of antigen-sensitized bentonite suspension was first carefully placed on the slide. An equal size drop of the test serum was then placed next to the antigen-sensitized bentonite suspension. The two test drops were thoroughly mixed by means of a glass rod. The slide was then gently rocked by hand for 5 min. All results along with negative and positive controls were read after every one minute with the naked eye, or under the low power of the microscope.

The entire range of reactions as (-); (±); (++); (+++) and (++++) were read according to the degree of flocculation. In a completely negative reaction, the suspension appeared as opaque and homogeneous, whereas a (++++) reaction always showed a well defined rim of flocculation.

3. Immunodiffusion (ID) Test

The test was performed as initially described by Ouchterlony (162). Two per cent ion agar along with 0.02 per cent sodium azide was dissolved in normal saline. The melted and sterilized agar was poured onto a glass slide to form a uniform 1 mm thick layer, which was then allowed to solidify. Another 2 mm thick layer was made by pouring and subsequently solidifying 1.5 per cent ion agar along with 0.02 per cent sodium azide in saline on top of the solidified layer. Two reagent wells (3 mm diameter each) at a distance of 5 mm apart were later cut in the upper layer of agar.

The central and peripheral wells were charged respectively with reacting antiserum and antigen samples. The glass slides were kept overnight in a moist chamber at room temperature and subsequently stored at 4°C for 72 hrs. All the precipitated bands were detectable within the 72 hrs of incubation. The photographs of the glass slides were taken after staining them with amido black dye.

4. Immunoelectrophoresis (IEP)

The immunoelectrophoresis was carried out according to the method of Scheidegger (163) and Garner and Gregory (164) in 1 per cent ion agar made in veronal buffer (0.05%, pH 8.6) on 2.5 X 7.5 cm slides. Two 12 mm apart holes of 3.0 mm each were cut in the layer of 1 per cent ion agar on the slides. A 50 X 1 mm trough in between the two holes was cut. The holes were then loaded with undiluted antigen preparation. The electrophoresis was carried out at 4°C at a potential gradient of 6 volts per slide for 5 hrs. After the electrophoretic separation of antigens, the trough was filled with antiserum and the slides were incubated for 72 hrs in a moist chamber.

5. Indirect Hemagglutination Test

Indirect hemagglutination test as described by Ming et al. (165) and later modified by Krupp (103,104) was used as follows:

Sheep blood was collected in an equal volume of Alsever's solution and the separated erythrocytes were washed three times in 0.15M sodium chloride solution by centrifugation for 5 min at 1000 x G at room temperature. The packed cells were chilled at 4°C in an ice bath and diluted to 2 per cent erythrocyte suspension with 1 per cent chilled glutaraldehyde salt solution. Glutaraldehyde salt solution was made by the addition of 4.0 ml of 25 per cent glutaraldehyde, 57.6 ml (0.15M) NaCl, 32.0 ml distilled water and 6.5 ml (0.15M) Na_2HPO_4. The solution was adjusted to pH 8.2

with 0.15M KH_2PO_4. The erythrocytes were then gently rotated at 4°C for 30 min. The erythrocytes were washed 5 times in normal saline by centrifugation, followed by 5 washings with distilled water. The gluteraldehyde-fixed erythrocytes were brought into a 2.5 per cent suspension by the addition of buffered saline (pH 7.2). If needed, the glutaraldehyde-fixed erythrocytes were brought into a 30 per cent suspension in distilled water, merthiolated (1:10,000) and stored at 4°C.

Equal volumes of 1:60,000 solution of tannic acid and 2.5 per cent suspension of washed glutaraldehyde-fixed and untreated sheep erythrocyte, both precooled at 4°C, were mixed and gently rotated at 4°C in an ice bath for 20 min. The cells were washed twice with phosphate buffered saline (7.2 pH) and were brought into a 2.5 per cent suspension in normal saline.

Sensitization: Equal volumes of 2.5 per cent glutaraldehyde-fixed, tanned erythrocytes and antigen dilutions (1:256) in PBS solution (pH 6.4) were mixed and incubated at 37°C for 20 min. The erythrocytes were then washed twice in 1 per cent agammaglobulin normal rabbit serum prepared in PBS of pH 7.2 (1 per cent ARS-PBS). All serum dilutions for the tests were made in 1 per cent ARS-PBS. After the cells were washed, they were suspended to obtain a 1 per cent suspension in 1 per cent ARS-PBS. For storage, the cells were frozen in dry ice-isopentane bath and kept at -70°C. Whenever needed, the cells were thawed in a 37°C water bath and used immediately.

Both gluteraldehyde-fixed and fresh untreated erythrocytes were sensitised with various dilutions of E. histolytica antigen. The optimal antigen dilution used for the detection of antibodies and for replication of titres was determined by making use of the negative and positive control sera in a preliminary titration. All tests were carried out in duplicate and, in parallel with untreated sheep erythrocytes. All sera samples for these tests were routinely collected from experimental rabbits, centrifuged at 3,000 X G for 20 min and stored at -20°C until used. The antiserum and the agammaglobulin rabbit serum samples used in the tests were routinely heated at 56°C for 30 min for inactivating complement.

Test Procedure: In the actual test, each well of the microtitre plate received 0.1 ml of serum dilution alongwith 0.05 ml of a 1 per cent suspension of gluteraldehyde-treated and antigen-sensitised erythrocytes. The plates were sealed with transparent gummed tape and gently rotated for 5 min at room temperature. The cells were allowed to pattern for several hrs and also the plates were shaken to redistribute the cells. The plates were then refrigerated overnight and the patterns were read and recorded next morning. The highest dilution of the test serum giving a positive carpet-like pattern was recorded as the end point of IHA titration.

The sequential sera samples and immunoglobulin fractions were also employed for the detection of antibody activity by means of the above described IHA tests.

6. Immunofluorescent Test

Serological detection and the confirmation of the presence of antibodies against E. histolytica antigen was done by the method of Boonpucknavig and Nairn (93), Maddison et al. (86) and Kane et al. (166).

The E. histolytica antigen dilution (1:16 dilution in distilled water) was placed in a 3 mm hole of a PTFF (Teflon)-coated slide. The antigen spots were air-dried and fixed with methanol for 1 min. The slides were then washed with phosphate buffer saline (pH 7.2) and then air-dried.

The following reactants were used for preparing the test slides:

1. Amebiasis immune sera
2. Normal serum as negative control
3. IgG fraction from immune serum
4. IgA fraction from immune serum
5. IgM fraction from immune serum

Doubling dilutions of the serum and immunoglobulin fractions were made with PBS as the diluent.

The diluted reactants were placed over the antigen spots (on the slides) and left undisturbed for 30 min. The slides were

later washed in PBS for 30 min. One drop of fluorescein-labeled monospecific anti-rabbit goat IgG at its optimal working dilution (1:5) was added to the reactant drop in each hole in different rows. The slides were again left undisturbed for 30 min in the humid chamber. Finally, the slides were washed thoroughly for at least one hr with buffered saline, blotted dry, mounted in buffered glycerol, and viewed under an ultraviolet light microscope.

The end point for each titration was recorded as the last dilution giving a definite apple green positive reaction, readily distinguishable from a negative control.

The sequential sera samples and the isolated immunoglobulin fractions were also employed in this test.

B. DETECTION OF GAMMA GLOBULIN

The detection of gamma globulin in sequential sera was done on 1 per cent agar-gel slides. The electrophoresis of 0.02 ml sequential sera was carried out for 2½ hrs in moist chamber for making comparisons with whole rabbit serum control. After electrophoresis, the slides were stained with 1 per cent amidoschwarz prepared in methanol. The gamma globulin levels of weekly sera samples were determined by comparing the color intensities.

C. ISOLATION OF GAMMA GLOBULIN

Blood samples from normal and immunized rabbits were obtained and kept at room temperature for about 1 hr and were then left overnight in the refrigerator. The clot was separated and the serum was removed by centrifugation. Human sera samples from a few positive cases of E. histolytica infections (extra intestinal) were also used for the isolation of globulins. The gamma globulin was isolated by the method of Heltman and Michael (167).

Gamma globulin was precipitated from the whole serum by adding 4C per cent ammonium sulphate. Precipitated samples were further refrigerated overnight for complete precipitation of gamma globulin. The precipitate was subsequently washed three times and dissolved in 0.15M NaCl. The precipitate was also dialysed against 0.15M NaCl until no barium sulphate was being precipitated on subsequent addition of barium chloride. The rabbit and human gamma globulins were also dialysed against 0.0175M phosphate buffer (pH 6.3). Salt fractionated gamma globulin was then used for further purification on DEAE-cellulose and Sephadex G-200 columns.

D. PURIFICATION OF IMMUNOGLOBULINS

1. DEAE-cellulose Column Chromatography

The separation of immunoglobulins was done according to the method of Cebra (168) and Sober (169).

Packing of the Column: DEAE-cellulose Whatman DE-11 (10 g) was suspended in 200 ml of 0.1N HCl and was stirred for about 30 min at room temperature. The suspension was filtered through Buchner funnel under partial pressure. The cellulose cake was removed and suspended in 200 ml 0.1N NaOH. The cellulose was stirred for about 10 min and was later filtered as described above. Filtered cellulose was then resuspended and washed several times in 200 ml of 0.1N NaOH. The cellulose was finally washed with 200 ml 0.0175M phosphate buffer (pH 6.3) till an ionic equilibrium between the cellulose and the effluent buffer was achieved. The cellulose was later resuspended in 200 ml of the above buffer and was stored in this form for subsequent use. Before filling the column, the cellulose was completely degerated for about half an hr by using a vacuum pump. The gel slurry was poured along a glass rod into a chromatographic column measuring 1.5 X 32 cm. After allowing the slurry to settle down, a pressure of 10 psi was applied to pack the column. A 3 to 5 cm column length left above the cellulose was filled with buffer solution. The column was also equilibrated with starting buffer.

Loading of the Column: Gamma globulins were dialysed in the starting buffer. Subsequently, the buffer solution above the cellulose bed was removed. Samples containing 12 mg gamma globulins were applied by taking care as not to disturb the column bed. A sufficient volume of buffer 3 - 5 ml above the bed was added before the column was connected to the gradient device for elution.

Elution: The column was connected to a device which maintained an ionic gradient difference of 0.0175M and 0.5175M respectively in the initial and final sodium phosphate buffer solutions. Both the buffer solutions were having the same pH value (6.3). The fractions were collected in 2 ml samples with an adjusted flow rate of 15 ml/hr. Protein concentration in each fraction was determined by Lowry's (153) method. The salt concentration in each fraction was calculated by using the following equation:

$$C = C_2 - (C_2 - C_1)\, e^{-V/Vo} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where C is the salt concentration of the protein fraction, C_1 is the salt concentration of the buffer in the mixing vessel; C_2 is the salt concentration of buffer in the second vessel connected to the mixing vessel; V is the volume of the eluent and Vo is the total volume in the mixing vessel which was maintained to 250 ml throughout the experiment. The volume of the buffer over the cellulose gel was maintained at 10.0 ml in all the experiments.

2. Sephadex G-200 Column Chromatography

Flodin and Killander (170) were the first to apply gel filtration to the separation of serum protein components. Later, Yap et al. (90) and Abioye et al. (91) employed this technic for the separation of immunoglobulins from amebiasis immune sera samples.

Sephadex G-200 was suspended in sodium phosphate buffer

(0.0175M, pH 6.3) and was allowed to swell for 5 hrs in a boiling water bath. The buffer was frequently changed during the swelling of the gel. The gel slurry prepared in the above buffer was deaerated for about half an hr by using vacuum pump. The slurry was poured into a column measuring 2.2 X 38 cm which also contained the above buffer. The packing was done at 10 psi and the flow rate was adjusted at 15 ml/hr. Before applying the samples, the void volume of the column was determined by using blue dextran 2000. Two ml samples of gamma globulin solution containing 50 mg of protein were applied to the column. Aliquot of 2 ml fractions were collected in a flow rate of 15 ml/hr. Protein concentration of each fraction was determined by the previously described Lowry's method.

3. Determination of Immunoglobulin Levels

Chromatographic column, filled with Sephadex G-200 gel, was used for separating serum immunoglobulins. Ten gram Sephadex G-200 was suspended in 0.1M PBS (pH 8.0) and allowed to swell for 5 hrs in a boiling water bath. The gel slurry also prepared in the same buffer was deaerated for about half an hr by using vacuum pump. The slurry was poured into a column measuring 2.1 X 76 cm. The column also contained the same buffer as was used earlier. The packing and eluting of the column was accomplished at a positive pressure of 10 psi in a flow rate adjusted at 20 ml/hr. The void volume of the column was determined by using blue dextran 2000. One ml aliquots of normal and immunised rabbit sera samples were applied to the column. Three ml fractions were collected, pooled

and lyophilised. Protein concentrations of the pooled fractions were determined by Lowry's method. The immunoglobulin levels in various fractions were determined by comparing the protein concentration and optical density of each peak.

II. STUDIES ON IMMUNOGLOBULINS

A. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide disc electrophoresis of immunoglobulins using 7.5 per cent acrylamide was carried out according to the method recommended by Davis (171). Glass tubes of suitable dimensions (length 75 mm and an internal diameter of 4 mm) were held vertically, with lower ends closed by stoppers. To each tube was added 2.0 ml of the small pore gel. The tubes were placed directly under a day-light fluorescent bulb. The bulb was so positioned that it remained about half to one inch above the tips of the gel tubes for 30 min during polymerization. The discontinuous buffer system of Davis (171) was used in which separation gel contained a Tris-HCl buffer of pH 8.9. A sample gel solution containing about 0.2 ml of large pore solution and 200 ug of immunoglobulins was thoroughly mixed and then added to the surface layer of small pore gel. The total amount of protein in the applied sample was not allowed to exceed beyond 200 ug. The photopolymerisation of the sample gel was achieved according to the above mentioned procedure.

The tubes with the sample gel upper most were inserted into

the grommets of the upper buffer reservoir. The reservoir was previously filled with stock buffer diluted ten-fold with distilled water. About 1 ml of a 0.001 per cent solution of bromophenol blue in water was then introduced and stirred into the upper buffer. The upper reservoir was then lowered in such a way that the bottoms of the gel tubes were immersed about one quarter of an inch in the buffer of the lower reservoir. The cathode was connected to the upper reservoir and the electrophoresis was carried out for 2 hrs using a current of 4 mA per tube until the dye (bromophenol blue) reached the bottom of the gel. The electrode buffer was made up of Tris-glycine at a pH 8.3. After the electrophoresis, the gels were removed and stained with 1 per cent Amido black in 7 per cent (V/V) acetic acid for 10 min. The gels were destained mechanically in 7 per cent (V/V) acetic acid until the destaining rendered the gel clear except at the protein bands.

B. CROSS REACTIVITY

The anti-immunoglobulins were raised in guinea pigs using Freund's complete adjuvant. The cross reactivity of all the immunoglobulins was checked against each specific anti-immunoglobulin sample by immunodiffusion according to the method described by Ouchterlony (162).

C. CARBOHYDRATE ESTIMATIONS

1. Estimations of Hexoses

The total hexoses were determined according to the method of

Winsler (172). The orcinol-sulphuric acid mixture was prepared by the addition of one volume of orcinol-(1.6 per cent in water) and 60 per cent of sulphuric acid in a ratio of 1:7.5 respectively. Portions of 1 ml (0.10 to 1.0 ml) standard mannose solution (50 mg/100 ml) were dispensed in 10 tubes. One tenth millilitre samples of IgG, IgM and IgA solutions were also dispensed in different tubes to be used as test samples. About 8.5 ml orcinol-sulphuric acid mixture was then added to these tubes and heated in a water bath at 80°C for 15 min. Water blanks were also included in the test. The tubes were later cooled in tap water and read at 505 nm.

2. N-acetylglucosamine Estimation

The glucosamine estimations were done according to the method of Reissig (173). Borate solution of 0.8M was made by dissolving 4.95 gm of boric acid in 40 ml water. This was kept stirring in 1N KOH till all the boric acid was brought in solution. The pH of the solution was adjusted at 9.1 in a volume of 100 ml. Ehrlich's reagent was made by dissolving 10 gm of p-dimethylaminoethyl benzaldehyde in 100 ml of glacial acetic acid containing 12.5 per cent (V/V) concentrated HCl. This stock solution while used was diluted 10 times.

The immunoglobulin samples were hydrolysed in 4N HCl at 100°C for 3.6 hrs. The blank and standard (N-acetylglucosamine 20 mg per 100 ml) tubes contained 0.1 to 1.0 ml solution samples.

The tubes were heated in a boiling water bath for 3 min, then cooled under tap water. Ehrlich's reagent (3 ml) was later added to each tube which was heated at 36-38°C for exactly 20 min. After cooling, the optical density was read at 585 nm.

3. Sialic Acid Estimation

The total sialic acid contents were determined according to the method of Svennerholm (174). Resorcinol solution was made by dissolving 0.2 gram in 10 ml water, 80 ml concentrated HCl and 0.25 ml of 0.1M copper sulphate. The volume was made up to 100 ml with the addition of distilled water. The reagent was prepared at least 4 hrs before using.

Isoamylalcohol was purified by mixing 1 liter with 200 ml of concentrated HCl and left for 2-3 days. The alcohol was washed about ten times each with 200 ml water, dried over anhydrous K_2CO_3 and then distilled. The fractions having boiling point of 130-135°C were collected. A 1 ml sample (hydrolyzed in 0.1M H_2SO_4 at 80°C for 60 min.) and 2.0 ml resorcinol reagent or blank reagent were mixed in glass stoppered test tubes. Standards with appropriate concentrations were prepared at the same time. The tubes were heated for 15 min in boiling water bath and then cooled in an ice bath to 20°C. The solutions were vigorously shaken with 5 ml of isoamylalcohol and cooled for 10 min in ice bath. The contents of the tubes were centrifuged for 1 min at 1000 rpm and isoamylalcohol layer was transferred to the cuvette. The readings were taken at 580 nm.

D. FLUORESCENCE MEASUREMENTS

Aminco-Bowman Spectrophotofluorometer with fused quartz cuvettes of 1.3 cm path length was employed for the measurements of fluorescence of immunoglobulins. Emission fluorescence spectra of immunoglobulins against E. histolytica antigen were measured. All the glassware was thoroughly washed in nitrating mixture prior to fluorescence measurements. All observations were taken at room temperature (22°C). The excitation was taken at 280 nm with sensitivity at 25 per cent while the length of the slit was 0.5 mm.

E. SPECTROPHOTOMETRIC MEASUREMENTS

Spectrophotometric measurements in the ultra-violet range were made by utilizing Beckman DK-2A ratio recording spectrophotometer with quartz cuvettes of 1 cm light path. All absorption measurements of immunoglobulins were carried out at room temperature (22°C).

F. MOLECULAR WEIGHT DETERMINATION OF IgG BY GEL FILTRATION

The molecular weight of the IgG was determined by gel filtration according to the method of Andrews (175) using Sephadex G-200 (2.1 X 54 cm) column. The column was equilibrated with 0.1M phosphate buffer, pH 7.5. A 8.3 mg sample of IgG was chromatographed on a column which was previously calibrated with five protein markers namely ovalbumin, α-chymotrypsinogen A, bovine

serum albumin (dimer), bovine serum albumin (monomer) and sheep IgG. The markers were of known molecular weights and Stoke's radius. The elution was performed with the same buffer as used above. Fraction samples of 3.0 ml each were collected at a flow rate of 20 ml/hr. Protein estimations were made at 700 nm using Lowry's method.

III. CELL-MEDIATED IMMUNE RESPONSES

A. IMMUNIZATION

The antigen used for immunization was obtained from Wellcome Research Laboratories, England. Each ampule contained approximately 200,000 amoba, freeze-dried from a small volume of 0.85 per cent saline. Each vial was reconstituted with 0.5 ml of normal saline and used as a stock solution. The antigen dilutions used for immunizing guinea pigs are given in Table II.

TABLE - II

Immunization Schedule in Guinea Pigs for CMI.

No.	Antigen in ml	Antigen in ug	Saline in ml	Freund's Adjuvant in ml	Total Volume in ml
1	0.0	00.00	0.5	0.5	1.0
2	0.1	35.25	0.4	0.5	1.0
3	0.2	70.50	0.3	0.5	1.0
4	0.4	141.00	0.1	0.5	1.0

The immunising animals were divided in four groups, each group consisting of four guinea pigs. One ml of this antigen preparation was injected to each guinea pig intradermally at 5 different sites on the shaved belly (0.2 ml at each site).

B. SKIN TEST

Eight days later, the guinea pigs were sensitized with 1:4 and 1:8 dilutions of ameba antigen given intradermally. Each dose contained 8.8 ug (1:4) and 4.4 ug (1:8) antigen in 0.1 ml injections given at two different sites on the back. Saline controls in equal volumes were also given in each animal. Skin reactions were observed immediately after administering the challenging dose and subsequently after 24, 48 and 72 hrs. The areas of the reaction sites were measured and recorded in both the experimental and control animals.

C. HISTOLOGICAL STUDY OF THE REACTION SITES

The sensitized animals showing positive dermal reactions were sacrificed and the excised skin from the reaction site was immediately fixed in 10 per cent formalin-saline solution. The excised tissue was later processed for histological studies. The slides were stained with iron-hematoxylin and eosin and later examined under the microscope.

D. MIGRATION INHIBITION REACTION

Fifteen days after immunisation, the guinea pigs from each

group were injected with 10 ml of sterile paraffin oil intraperitoneally for obtaining the peritoneal exudate. Three days later, the peritoneal exudate was obtained after injecting 10 ml of heparinized Hank's balanced salt solution (HBSS) intraperitoneally. The blood was also taken on the same day, directly from the heart, for humoral antibody study. The paraffin oil layer was removed from the peritoneal exudate and washed three times in HBSS at 800 rpm for 5 min. The cells were adjusted at 5×10^7 cells/ml in TC-199 containing 10 per cent heat inactivated horse serum.

The animals were tested individually from each group. For each animal, 90 ul of cell suspension and 10 ul of antigen solution (containing 0.83 ug of antigen) were mixed in separate tubes. The controls received an equal volume (10 ul) of medium containing 10 per cent heat inactivated horse serum. This suspension was incubated at 37°C for 30 min in a moist incubator.

1. Capillary Method

Capillary migration inhibition test was carried out according to the method of David et al. (176,177). Incubated cells were taken in capillary tubes (2×10^6 cells) and sealed at one end with plasticine and centrifuged for 5 min at 1000 rpm. The tubes were broken at packed cell-fluid interface and the portion containing cells was fixed with silicon in the migrating chamber containing TC-199 medium. The chamber was covered with a cover slip and sealed with silicon. Care was taken to avoid air bubbles in the chamber. The migrating

chambers were incubated at 37°C for 20 hrs in an incubator with 5 per cent CO_2 and humidity.

On incubation, the cells migrated out fan-wise on the bottom of the chamber. The microscopic image of cell migration area was measured by projecting with a Bausch and Lomb Prism onto a drawing paper. The outline of this migration was drawn and cut with the scissors. The migrating area for each test was obtained by calculating the mean weight of the migrating area from the test and the control tubes. The following formula was used to express the extent of migration of cells from the peritoneal exudates:

Per cent of Migration Inhibition =

$$1 - \frac{\text{Weight of migration area in the presence of antigen}}{\text{Weight of migration area in control}} \times 100$$

The average area was calculated from atleast four capillary tubes.

2. Agarose Plate Technic

Migration inhibition test on agarose plates was carried out according to the method of Clausen (136). Agarose medium was prepared by dissolving 2 gram of agarose (Litex, Glostrup, Denmark) in distilled water. The solution was cooled to 47°C and mixed with inactivated horse serum at the same temperature. The agarose solution also contained water and a 10-fold concentration of tissue culture

medium (TC-199). The final solution contained 1 per cent agarose and 10 per cent serum in single strength TC-199. Penicillin and streptomycin was added to give a penicillin concentration of 66 IU/ml and a streptomycin concentration of 66 ug/ml. Sodium bicarbonate solution (0.1M) was added so that the pH of the agarose medium after incubation in 5 per cent Co_2 in air was between 7.2 to 7.4.

Five ml of agarose-serum-TC medium 199 was poured into disposable 50 mm plastic petri dishes (Millipore Filter Corp., Bedford, Mass). When the gel had formed, eight holes with a diameter of 2.3 mm were cut in each agarose plate with a stainless steel punch.

Seven microlitre sensitized peritoneal exudate and control samples containing 1.5×10^6 cells were placed in each hole within the agarose medium. About six to eight cultures were made for each group of cell suspension.

The agarose plates were incubated at 37°C in 5 per cent CO_2 within a 100 per cent humid atmosphere for 24 hrs. The cells and the agarose medium were fixed in 7.5 per cent glutaraldehyde for 30 min. The agarose was removed from the dishes and the cells adhering to the bottom were rinsed with distilled water and, dried. The migration areas were studied under a projection microscope and measured by planimetry. The migration inhibition was calculated

as follows:

Per cent of Migration Inhibition =

$$1 - \frac{\text{Mean migration area in presence of antigen}}{\text{Mean migration area in controls}} \times 100$$

CHAPTER - IV

R E S U L T S

A. AXENIC CULTURE OF AMEBA

Entamoeba histolytica (Strain NIH: 200) cells were axenically grown in Diamond's TP-S-1-monophasic liquid medium. The cultures were generally seeded with 15,000 - 20,000 amebae/ml in a volume of 1.0 ml inoculum/12.5 ml medium. The total cell count after 96 hrs of incubation gave an average of 2×10^5 amebae/ml of medium. All amebae counts were taken by Neubauer hemocytometer.

B. ANTIGEN PREPARATION

Water-soluble antigenic extracts of pooled amebae were prepared according to the method of Kessel et al. (113). The cells were washed two times in normal saline. One ml packed cells were diluted 10 times and passed through a syringe after freezing and thawing several times, and centrifuged at 20,000 X G for 20 min. The protein value of the soluble antigenic extract was found to be 3.6 mg/ml.

I. DETECTION OF ANTIAMEBIC ANTIBODIES

The rabbits immunized with E. histolytica antigen were used for the evaluation of humoral immune responses. The humoral immune responses were evaluated by detecting antibodies in indirect hemagglutination, bentonite slide flocculation, precipitin titration, immunodiffusion, immunofluorescent and immunoelectrophoresis tests.

1. Precipitin Ring Test

In the precipitin titration, a positive test was indicated by the formation of a precipitin ring at the interface of the two reactants. The highest dilution of antigen which gave a positive reaction against antiserum was 1:256. This test was primarily used for obtaining an optimal ratio of the two reactants (antigen and antibody) for their subsequent use in various antigen-antibody reactions.

2. Bentonite Slide Flocculation Test

Sera samples from normal and immunized rabbits were tested for the presence of antibody activity by means of BSF tests. These tests were read on the basis of the degree of flocculation. The flocculation range from (± to ++++) was taken as indicative of the amount of flocculating antibodies. All immunized rabbit sera samples showed a flocculation of (++++) grade. The plus four reaction was taken as an index of complete flocculation on the slide. The highest titre of the immune serum on this test was recorded up to 1:160. No flocculation was recorded in control rabbit serum.

3. Immunodiffusion Test

Specific antibodies were detectable from immune rabbit sera in this test. Generally, 5 well defined bands were obtainable in

immune sera samples after 72 hrs of incubation against whole antigen. A sample of 1:256 antigen dilution was used for the antigen-antibody reaction in this test. The results of ID test are shown in Figure 1.

The isolated immunoglobulin fractions were also used in this test. IgG fraction gave four bands, while the IgM fraction only two. The antiamebic antibody activity was not found in IgA fraction. The resolution of the isolated fractions in the ID tests are depicted in Figure 2.

4. Immunoelectrophoresis Test

The results of antibody analyses in immunoelectrophoresis were not very different from those of immunodiffusion. The immune rabbit sera showed antibody activity against the optimal antigen dilution (1:256). The immune rabbit serum gave 5 bands in this test. A representative immunoelectrophoretic pattern as obtained in the above tests is shown in Figure 3.

The immunoglobulin fractions (IgG and IgM) from immune serum were also used for detecting the antibody activity. IgG fraction showed the presence of 4 precipitin bands in immunoelectrophoretic separations. IgM fraction gave only 2 bands. Fraction IgA from DEAE-cellulose was negative for the presence of antibody activity. The electrophoretic pattern of the various fractions is represented in Figure 4. The tabulated results of immunodiffusion and immunoelectrophoresis tests are shown in Table III.

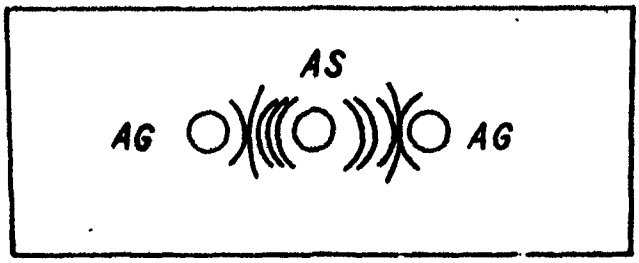


Figure 1. Precipitin bands obtained from rabbit immune serum against E. histolytica antigen.

AG: Antigen; AS: Antiserum.

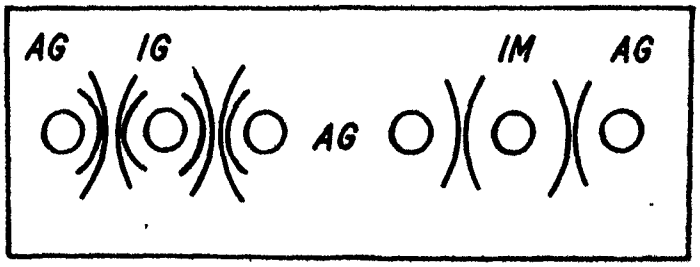


Figure 2. Precipitin bands obtained from chromatographically isolated fractions from rabbit immune serum.

AG: Antigen; IG: Immunoglobulin G; IM: Immunoglobulin M.

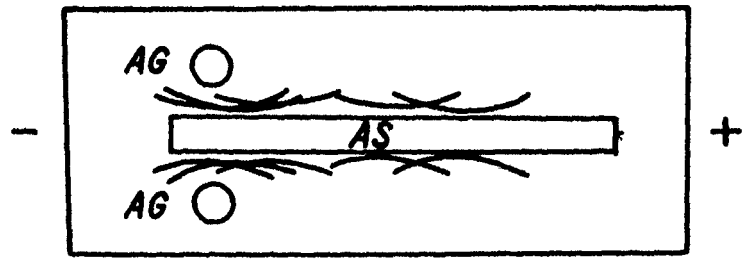


Figure 3. Immunoelectrophoretic pattern of E. histolytica axenic antigen (AG) developed against whole serum (AS).

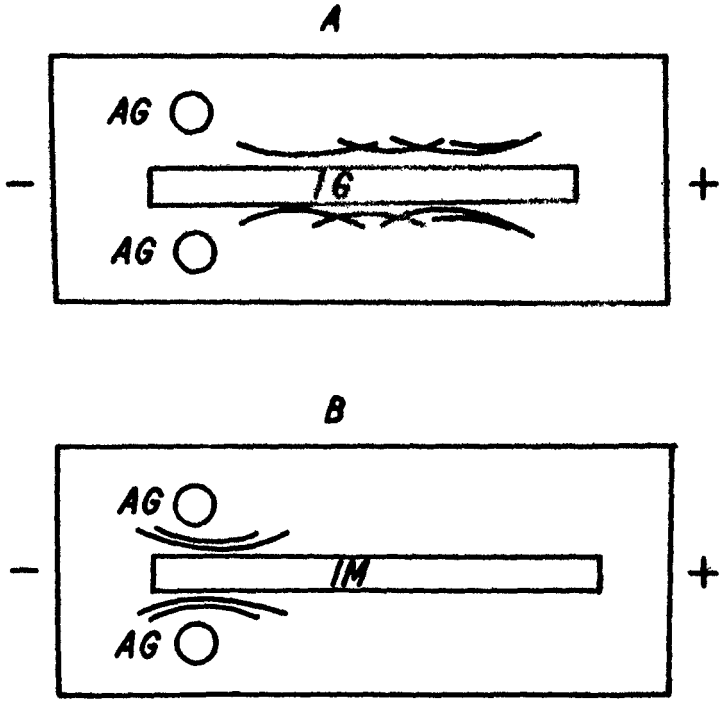


Figure 4. Immunoelectrophoretic patterns of E. histolytica axenic antigen (AG) developed against chromatographically isolated fractions: A) IgG fraction and B) IgM fraction.

TABLE - III

Reactivity of Immune and Control Serum.

Serum	Precipitin Titre	BSF	IHA Titre	FA Titre	ID	IEP
[redacted]	1:256	1:160	1:4096	1:8192	5	5
[redacted]	-	-	-	-	-	-

BSF : Bentonite slide flocculation test.

IHA : Indirect hemagglutination test.

FA : Fluorescent antibody test.

ID : Immunodiffusion test.

IEP : Immunoelectrophoresis test.

5. Indirect Hemagglutination Test

Preliminary box type titrations used for determining the optimal antigen ratio gave 1:256 dilution as the highest titre showing detectable precipitation. This antigen dilution (1:256) was then used for sensitization of sheep erythrocytes. The highest titre of the immunized serum giving a positive IHA test was found to be 1:4096. The isolated IgG and IgM fractions from the pooled serum also showed IHA titre values as 1:4096 and 1:1024 respectively. The IHA activity was completely absent in IgA fraction.

6. Immunofluorescent Test

Specific antibody activity was also determined by assessing the fluorescence in the value range of (+ to ++++). The highest serum dilution giving specific and an unambiguous immune fluorescence was taken as the end point. Samples of immune serum and the isolated IgG fractions when allowed to react with FITC anti-rabbit goat IgG showed greenish fluorescence against a reddish background. The fluorescence was obtained after counter-staining the test slides with Evans Blue. The highest titre of the antiserum showing a positive reaction was 1:8192. No fluorescence was observed on the slides which were layered with the isolated IgM and IgA fractions. Control slides smeared with non-immune serum did not show any fluorescence. The results of SSF, precipitin, IHA and immunofluorescence tests are tabulated in Table IV.

TABLE - IV

Reactivity of the Isolated Immunoglobulins.

Immunoglobulin	TITRE			
	ID	IEP	IHA	FA
IgG	4	5	1:4096	1:8192
IgM	2	2	1:1024	-
IgA	-	-	-	-

ID : Immunodiffusion test.
IEP : Immunoelectrophoresis test.
IHA : Indirect hemagglutination test.
FA : Fluorescent antibody test.

A. PURIFICATION OF IMMUNOGLOBULINS

1. Fractionation on DEAE-cellulose

A single symmetrical peak was obtained when whole non-immunized rabbit and human serum (Figure 5) or the gamma globulin from normal rabbit or immune human serum (Figure 6) were chromatographed on DEAE-cellulose columns in the absence of any salt gradient. Ammonium sulphate (40%) fractionated rabbit and human gamma globulin, or whole serum, resolved into three well defined peaks in a linear salt gradient (Figures 7 and 8). Figure 9 illustrates the elution profile

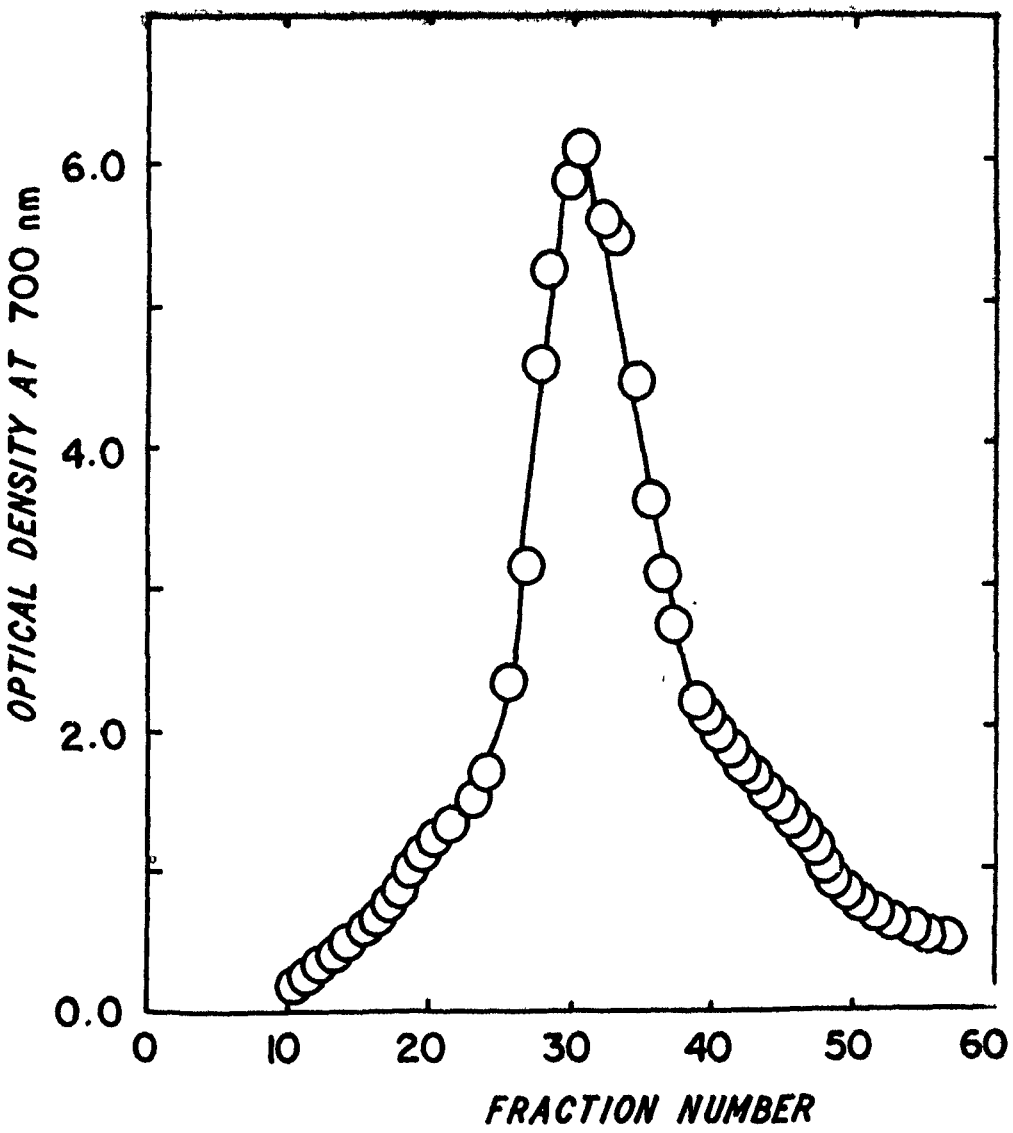


Figure 5. Chromatography of normal rabbit serum on DEAE-cellulose column.

Experimental conditions: One ml serum containing 76.5 mg protein was applied to the column (3 x 38 cm) and eluted with phosphate buffer (pH 6.3; molarity 0.0175) at a flow rate of 30 ml/hr.

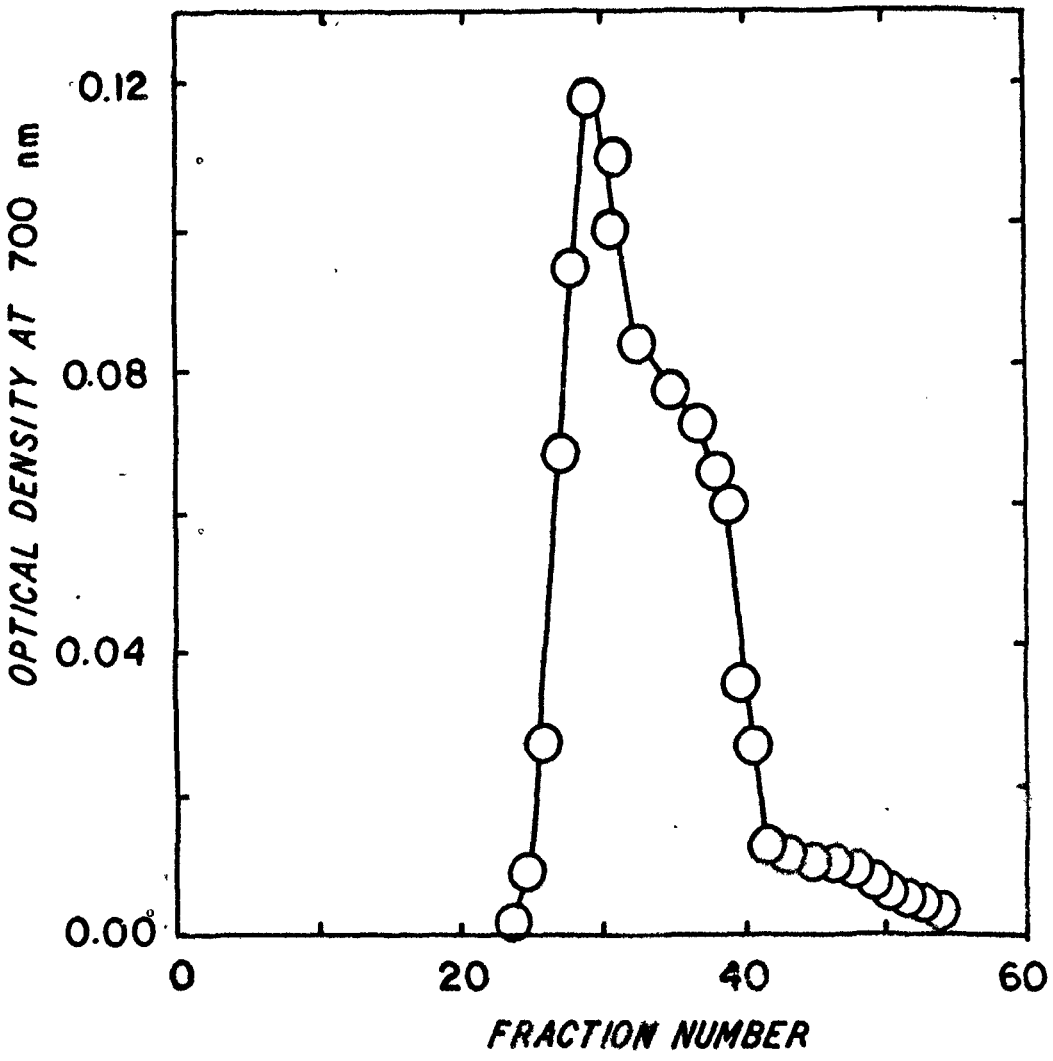


Figure 6. Chromatography of gamma globulin from human serum on DEAE-cellulose column.

Experimental conditions: 17.06 mg of protein was applied to the column (3 x 38 cm) and eluted with phosphate buffer (pH 6.3; molarity 0.0175) at a flow rate of 30 ml/hr.

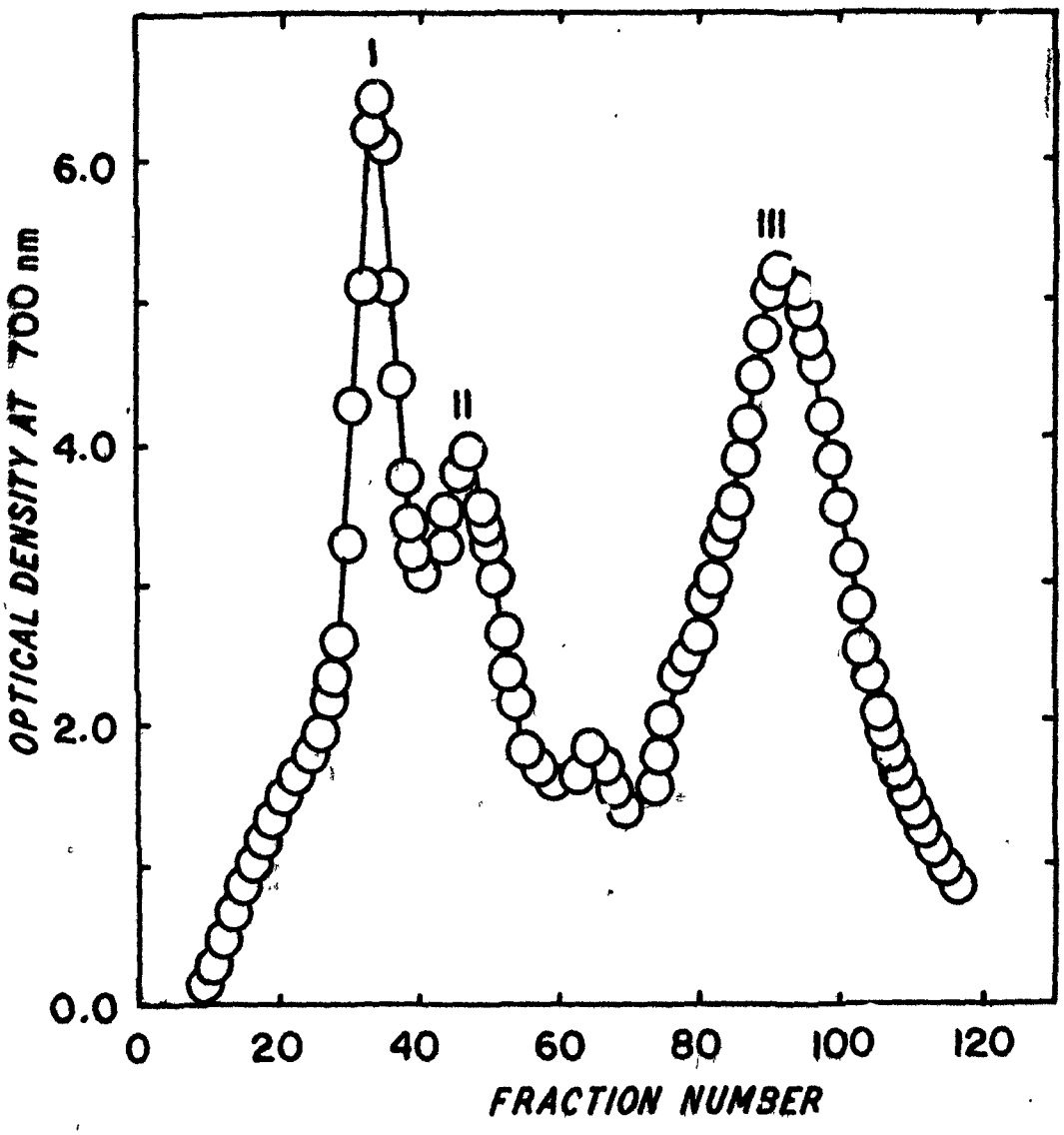


Figure 7. Chromatography of ammonium sulphate fractionated normal rabbit gamma globulin on DEAE-cellulose column.

Experimental conditions: Three ml sample containing 45 mg protein was applied to the column (1.5 x 34 cm) and eluted with phosphate buffer (pH 6.3; molarity range 0.0175 to 0.5175) at a flow rate of 15 ml/hr.

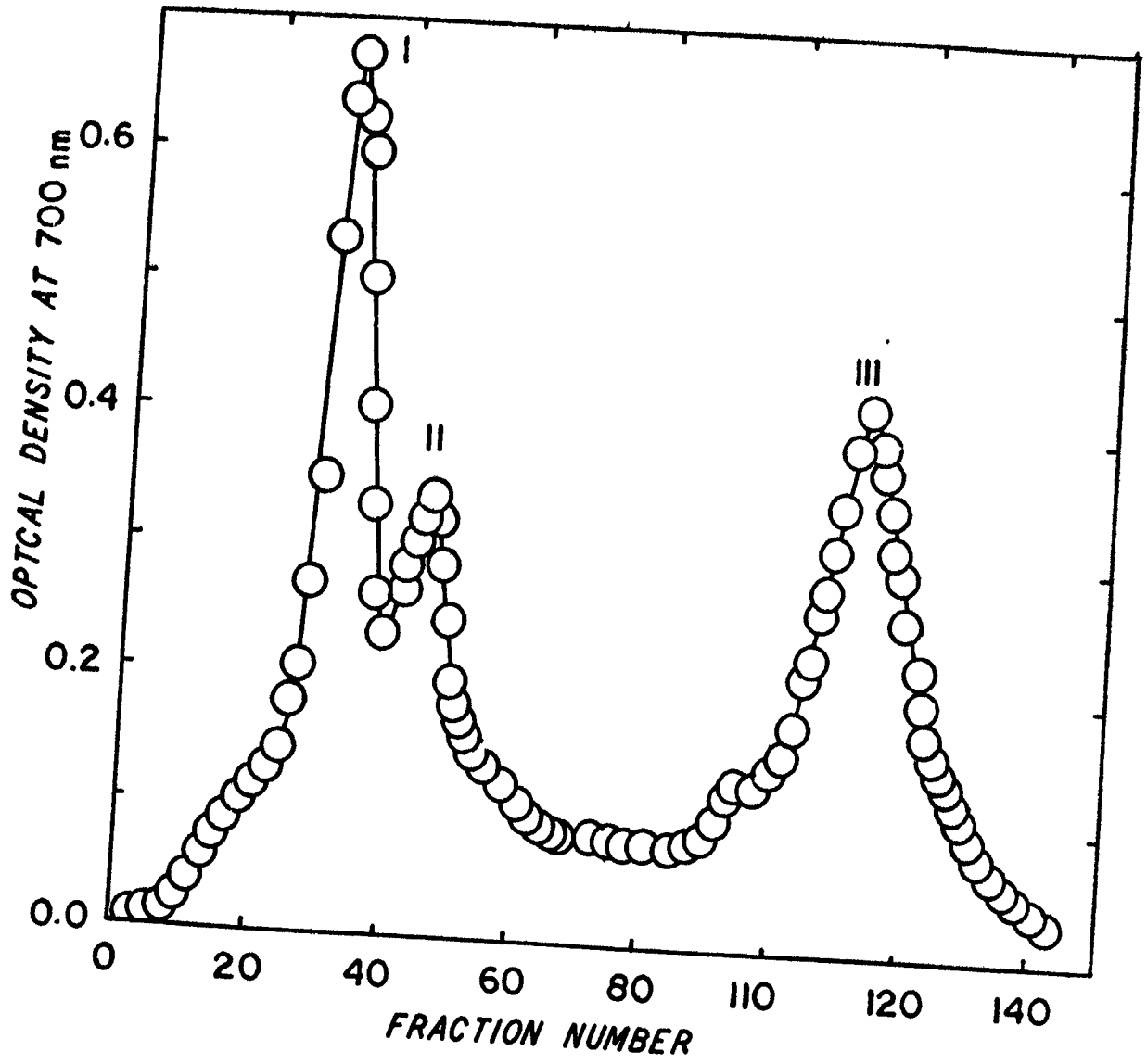


Figure 8. Chromatographic pattern of immune rabbit gamma globulin on DEAE-cellulose column.

Experimental conditions: Two ml sample containing 32 mg protein was applied to the column (1.5 x 32 cm) and eluted with phosphate buffer (pH 6.3; molarity range 0.0175 to 0.5175) at a flow rate of 15 ml/hr.

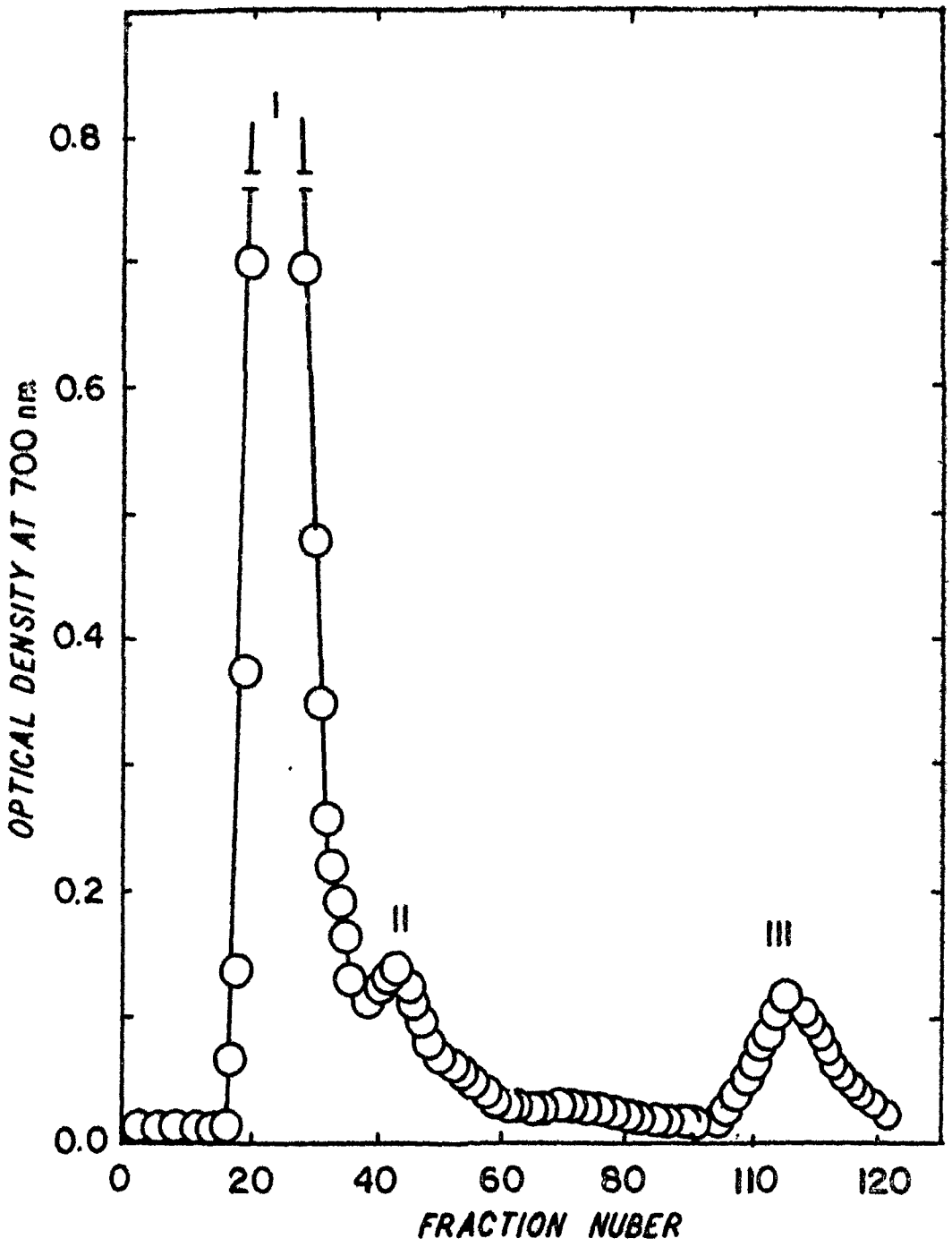


Figure 9. Chromatographic pattern of commercial human gamma globulin on DEAE-cellulose column.

Experimental conditions: Two ml sample containing 16 mg protein was applied to the column (1.5 x 32 cm) and eluted with phosphate buffer (pH 6.3; molarity range 0.0175 to 0.5175) at a flow rate of 15 ml/hr.

of a commercial preparation of human gamma globulin in a linear salt gradient. The three identical peaks I, II and III, as tested against rabbit monospecific anti-immunoglobulin sera, were found corresponding to IgG, IgA and IgM respectively (178).

2. Fractionation on Sephadex G-200

Ammonium sulphate (40%) fractionated gamma globulins from normal and hyperimmune rabbit serum resolved into three well defined peaks (Figures 10 and 11). Peak I and II represent IgM and IgG respectively. Peak III which has a mixture of IgG and IgA, infact, contains major part of albumin. Each of the three peaks were also separately rechromatographed for checking the homogeneity of the immunoglobulin molecule.

B. STUDIES ON EXPERIMENTAL SERA SAMPLES

1. Detection of Gamma Globulins in Agar Gel

The immunized sera samples when subjected to electrophoresis in a volume of 0.02 ml in one per cent agar gel showed a considerable increase in gamma globulin levels on the cathodic side of the slide. The actual increase in the gamma globulin levels was indicated by the size and the intensity of the stained area. The non-immunized serum was used as a control. The total level of gamma globulin appears to be more intense in the third and fourth week sera samples as shown in Figures 12a and 12b.

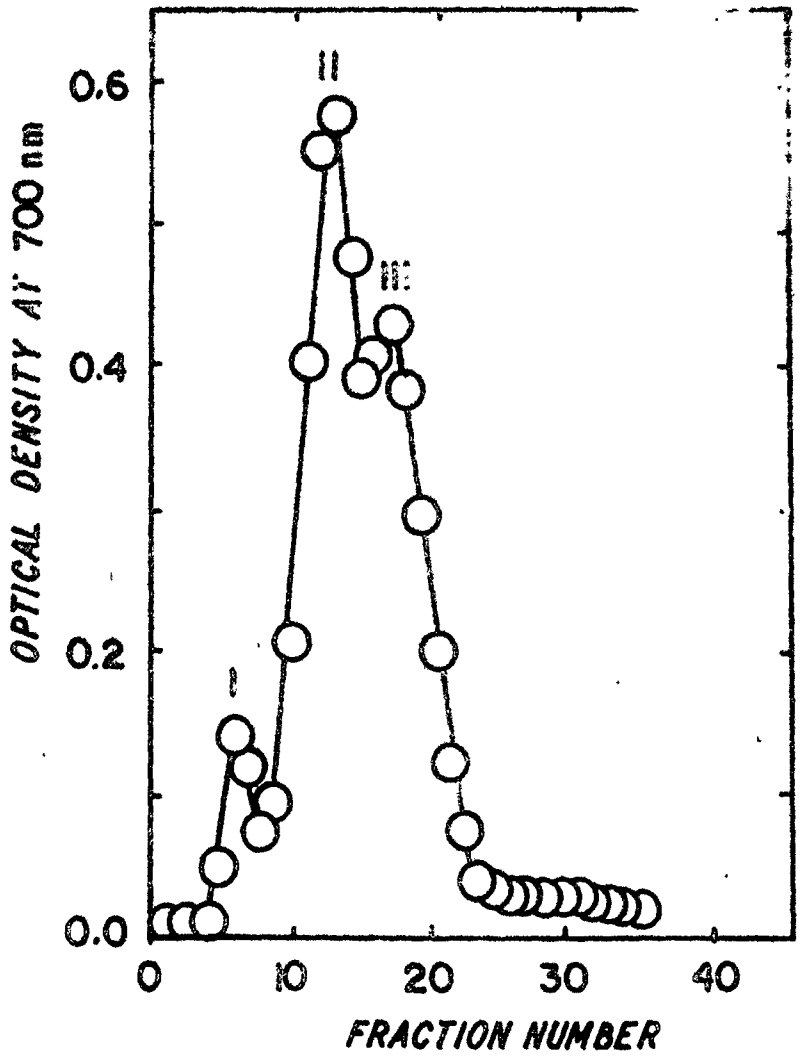


Figure 10. Chromatographic pattern of normal rabbit gamma globulin on Sephadex G-200.

Experimental conditions: A column measuring 2.2 x 36.5 cm was equilibrated with phosphate buffer of pH 6.3 and molarity 0.1. About 50 mg protein was applied and eluted at a flow rate of 15 ml/hr.

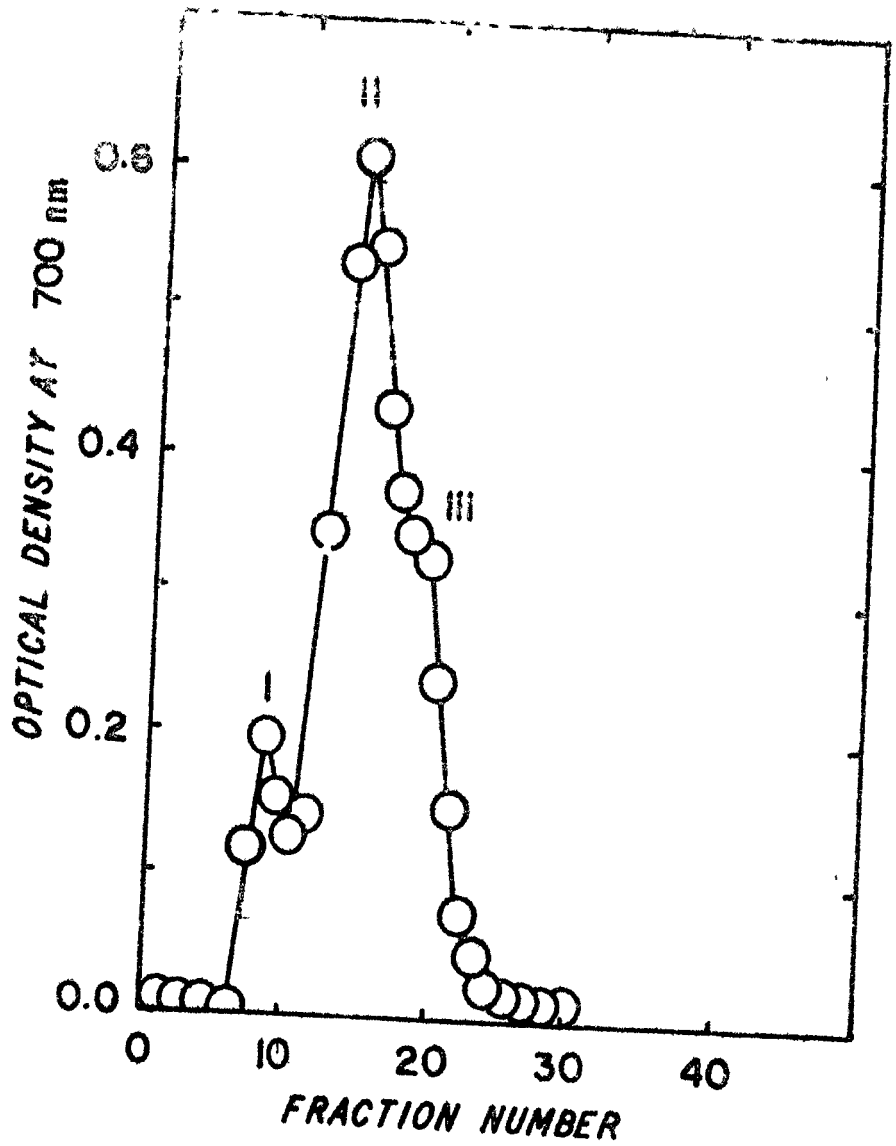


Figure 11. Chromatographic pattern of immune rabbit gamma globulin on Sephadex G-200.

Experimental conditions: A column measuring 2.2 x 36 cm was equilibrated with phosphate buffer of pH 6.3 and molarity 0.1. About 50 mg of protein was applied and eluted at a flow rate of 15 ml/hr.

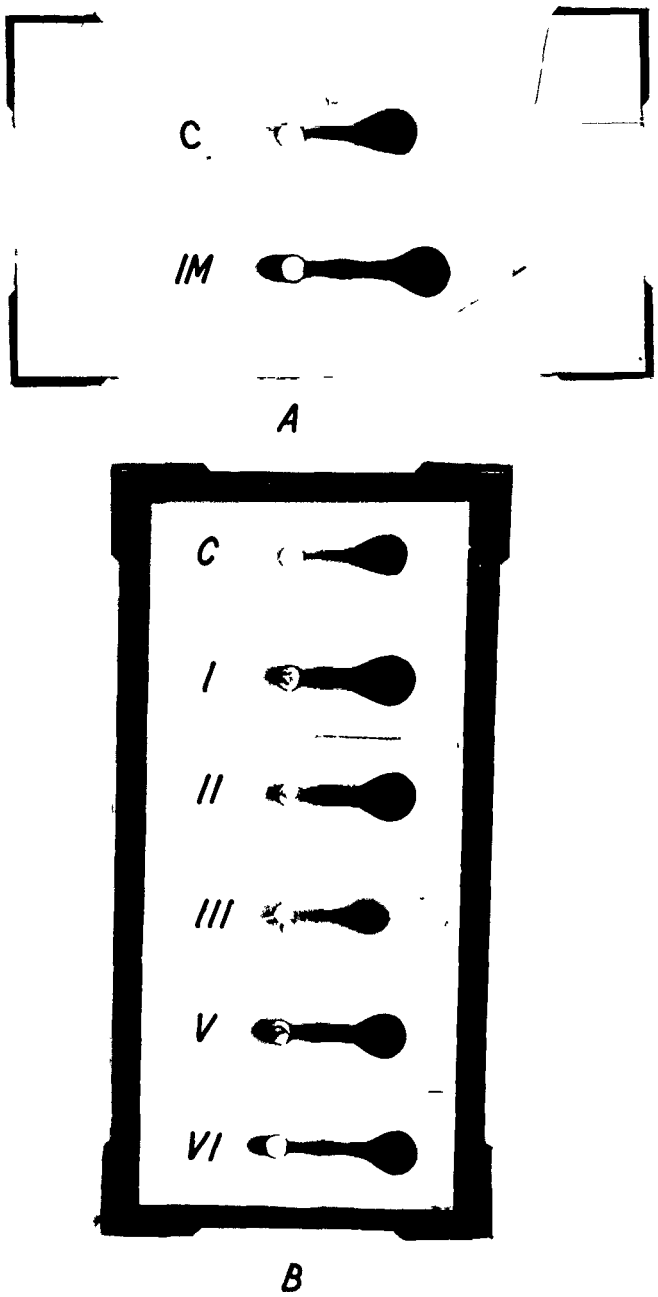


Figure 12. Electrophoresis showing a quantitative difference in the amount of gamma globulins between a) control and immune rabbit serum and, b) the primary and secondary response sera.

(C: control; I, II, III, V and VI week antiamebic sera samples).

2. Determination of Immunoglobulin Levels

The immunoglobulin levels from the normal and hyper-immune rabbit serum were determined by passing an equal volume of serum on Sephadex G-200 column. Three peaks were obtained after chromatography. Peak I represents the IgM, peak II a mixture of IgG and IgA, and peak III is that of IgG, IgA and albumin. Immunoglobulin concentrations were established in both the animal groups by estimating the protein concentration in the pooled fraction of each immunoglobulin separately. Such estimations were also made by comparing the absorption maxima of each peak separately (Figures 13 and 14). The immunoglobulin levels, following isolation on Sephadex G-200, are depicted in Table V and Figure 15.

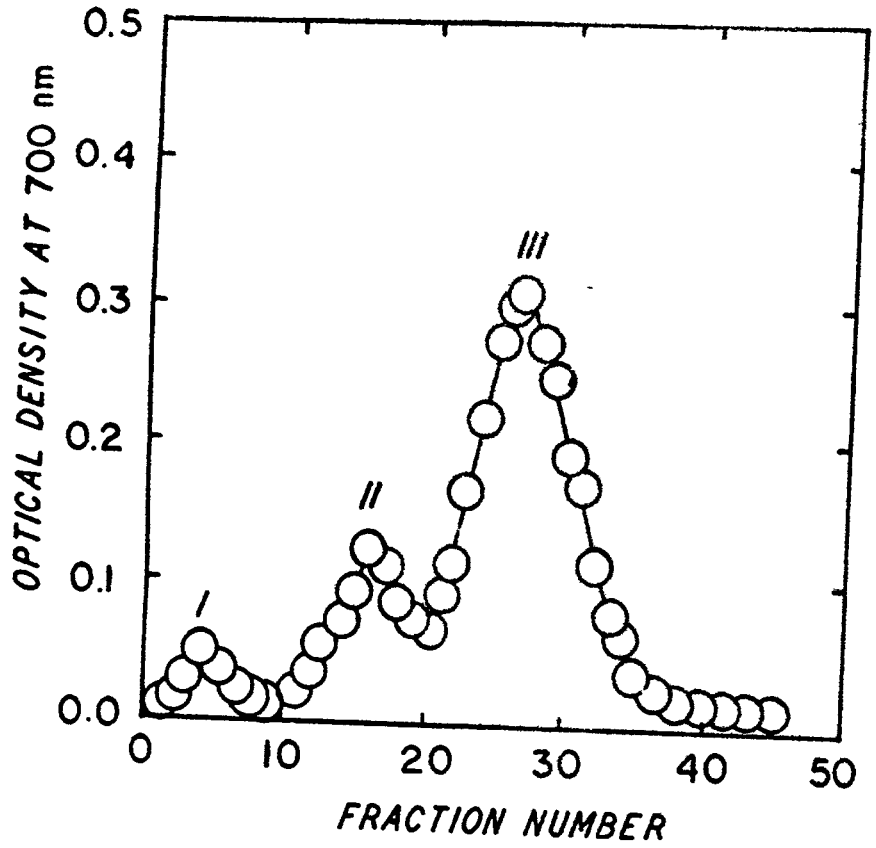
3. IHA Titre in Sequential Sera

The sheep red blood cells sensitized in the previous experiment were used in this test. The IHA titre values from primary and secondary response sera were used for making a quantitative estimation of the specific hemagglutinins in the sequential sera samples (Figure 16). The IHA activity in I, II and III week sera appears to be more or less constant (titre 1:128). After booster injections, the IHA activity was markedly increased (titre 1:1024) in the V week. Subsequently, it decreased to a titre of 1:512 by the VI week.

TABLE - V

Protein Concentration in Isolated Immunoglobulins from Sequential Sera (mg/ml).

Immunoglobulins.	Normal Initial	WEEKS					
		I	II	III	IV	V	VI
IgM	1.67 ± 0.096	3.79 ± 1.25	3.60 ± 0.21	2.00 ± 0.561	1.98 ± 0.49	4.95 ± 0.51	3.50 ± 0.23
IgG	7.82 ± 0.238	11.77 ± 1.17	13.98 ± 0.23	13.77 ± 1.19	13.00 ± 0.56	14.96 ± 0.28	13.90 ± 0.462



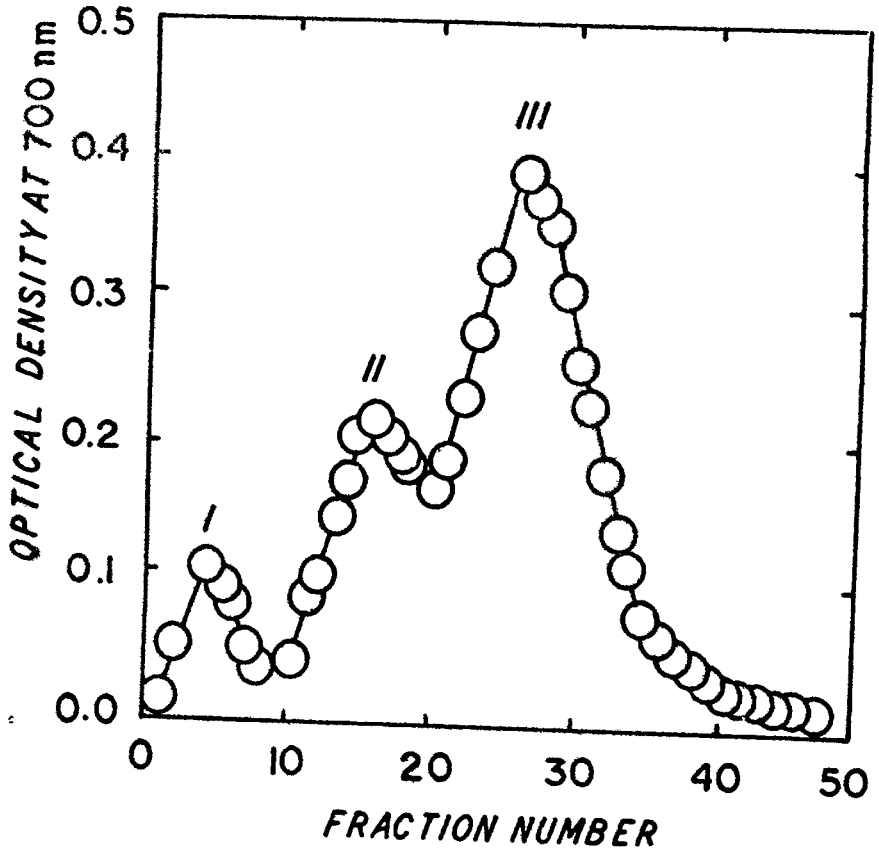


Figure 13 and 14. Chromatographic pattern of normal and immune rabbit serum on Sephadex G-200. Experimental conditions: A column measuring 2.1 x 79.5 cm was equilibrated with phosphate buffer of pH 8.0 and molarity 0.3. One ml serum was applied and eluted at a flow rate of 15 ml/hr.

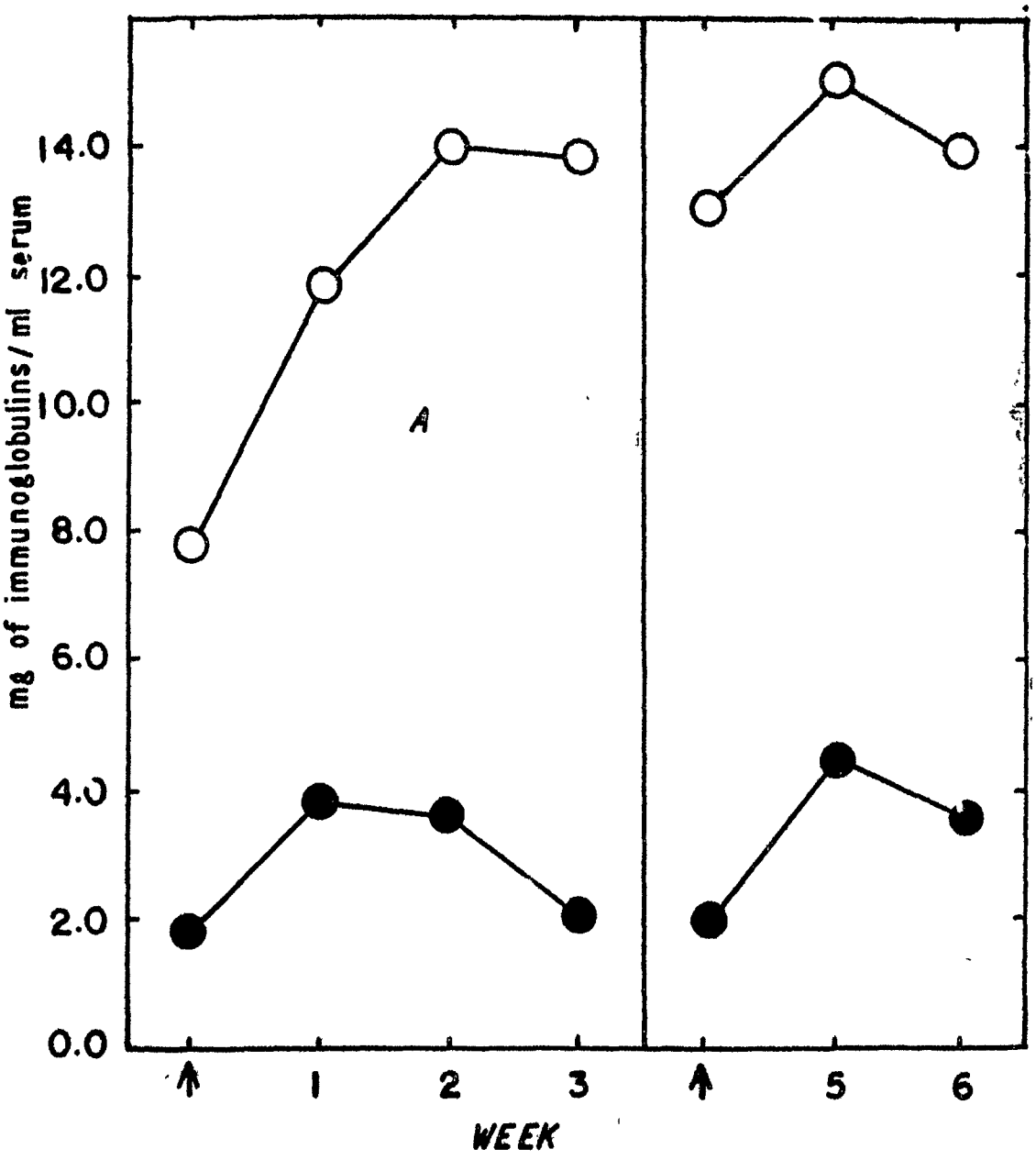


Figure 15. Immunoglobulin levels of the sequential sera samples on Sephadex G-200 columns.

A: Primary response serum; B: Secondary response serum; (O—O , IgG; ●—● , IgM).

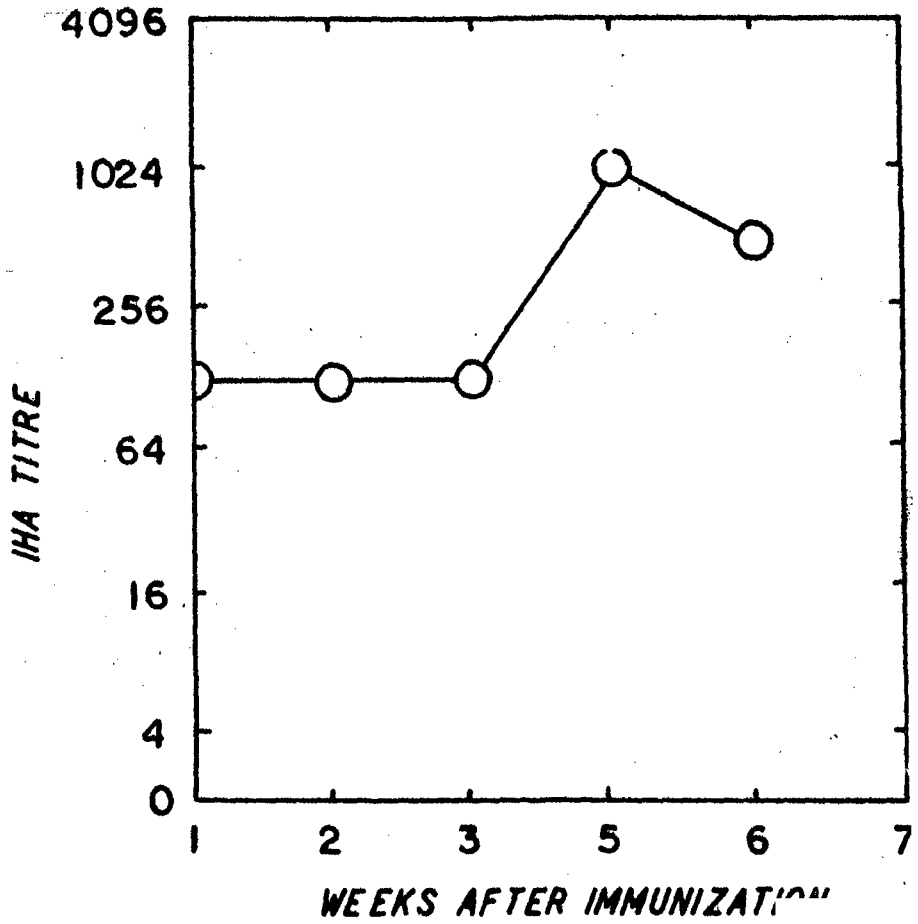


Figure 16. Indirect hemagglutination titres of sequential sera samples.

4. FAT Titre in Sequential Sera

The FAT titre values as obtained from sequential sera samples are given in Figure 17. The FAT titre (1:16,384) from the secondary response serum in the fifth week was found to be higher than that of primary response serum.

II. STUDIES ON IMMUNOGLOBULINS

A. POLYACRYLAMIDE GEL ELECTROPHORESIS

The isolated immunoglobulins from DEAE-cellulose were further passed through Sephadex G-200 for checking the homogeneity of the various fractions. The homogeneous peaks obtained from Sephadex G-200 column were then used for polyacrylamide gel electrophoretic analyses. Figure 18 shows the electrophorotogram of IgM, IgG and IgA fractions from hyperimmune rabbit serum. The isolated IgG fraction was completely pure. It resolved into a single sharp band in PAG. This single band was comparable and similar to the one obtained for standard IgG of rabbit. The IgM fraction gave two bands, one major and one minor and was comparable to the standard IgM of rabbit. The separation of IgA was not quite satisfactory in PAG electrophoresis.

The antisera against the isolated immunoglobulins were also raised for studying the cross reactivity, if any.

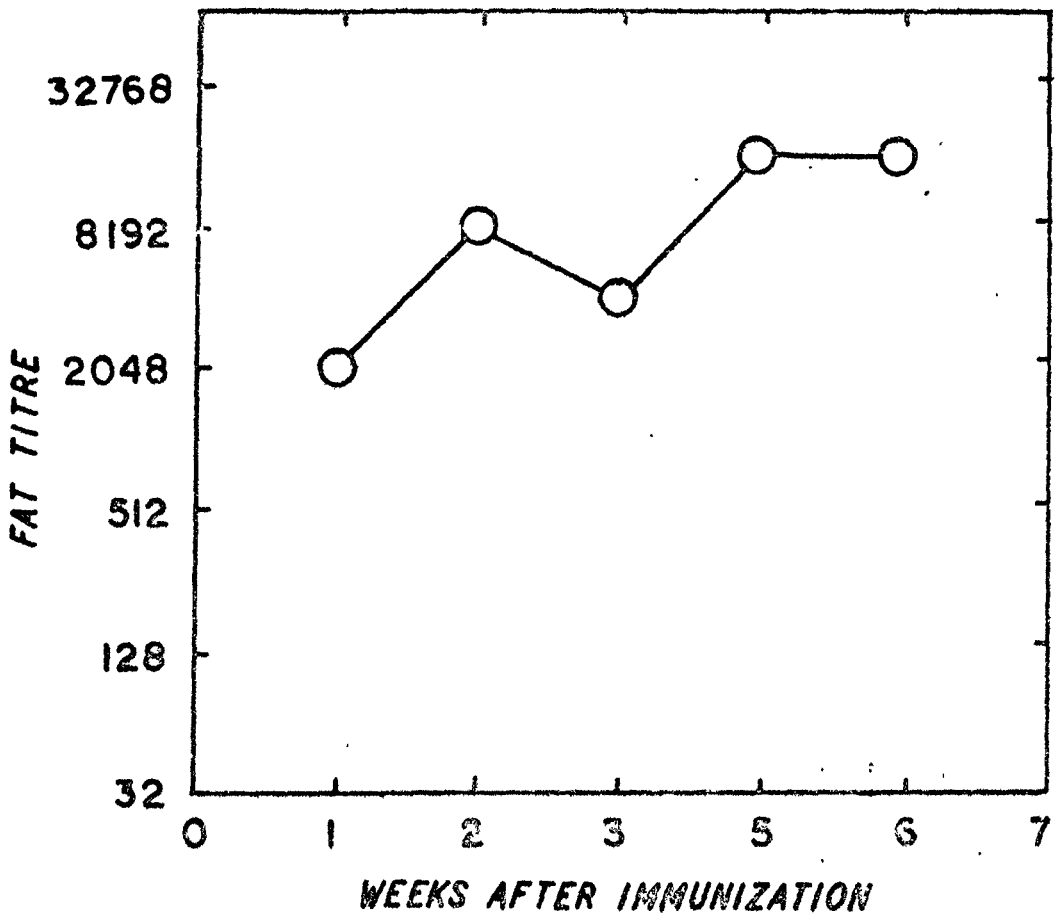


Figure 17. Fluorescent antibody test titres of sequential sera samples.

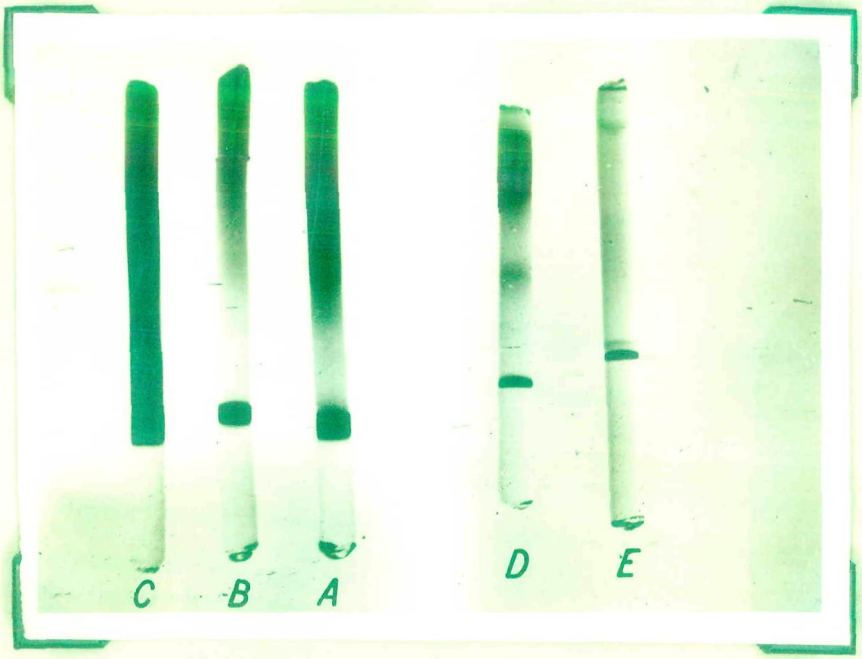


Figure 18. Diagramatic representation of the disc electrophoretic patterns of isolated immunoglobulins, (a) IgM; (b) IgG and (c) IgA. The isolated fractions were run in parallel with commercially obtained samples of immunoglobulins, (d) IgG and (e) IgM.

B. CROSS REACTIVITY

The homogeneous preparation, obtained after DEAE-cellulose and Sephadex G-200 chromatographic separations, was used for the immunization of guinea pigs. The monospecific antiserum obtained against each immunoglobulin was used for checking the homogeneity of the isolated fractions in immunodiffusion tests. No cross reactions, whatsoever, were found against each other. The immunoglobulins isolated from Sephadex G-200 chromatographic separations were found to cross-react nonspecifically with the antisera raised against other immunoglobulins. The cross-reactivity results are shown in Figure 19.

C. CARBOHYDRATE ESTIMATIONS

Carbohydrate estimations were also made in chromatographically isolated fractions from DEAE-cellulose and Sephadex G-200 columns. Such estimations, based on total hexoses, glucosamine and sialic acid contents, are shown in Table VI.

TABLE - VI

Carbohydrate Composition of Immunoglobulins (g/100 g of protein).

Immunoglobulins	Hexoses	N-acetylglucosamine	Sialic Acid
IgG	1.06	0.79	2.56
IgM	5.25	0.96	2.34
IgA	4.70	0.32	0.53

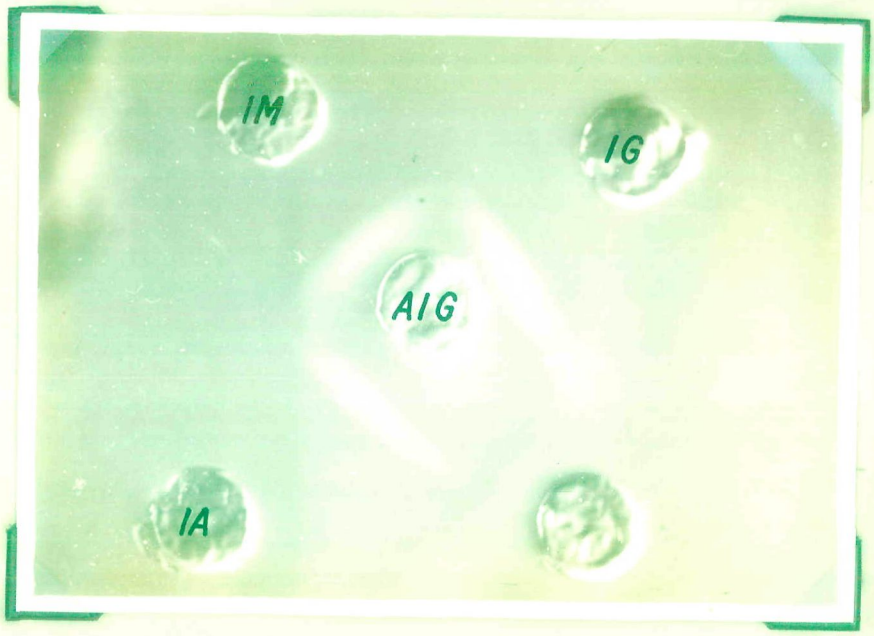


Figure 19. Cross reactivity of isolated fractions (IgG, IgM and IgA) against anti-IgG serum raised in guinea pigs.
(IM: IgM; IG: IgG; IA: IgA; AIG: anti-IgG).

D. FLUORESCENT SPECTRA MEASUREMENTS

Fluorescence emission maxima of IgG, IgM and IgA in 0.1M sodium phosphate buffer, pH 8.0, were found to be 350 nm. The fluorescence characteristics of the proteins were identical to the proteins containing tryptophenyl residues (179). The emission spectra are shown in Figures 20, 21 and 22.

E. UV ABSORPTION

The UV absorption spectra of IgG, IgM and IgA are further shown in Figure 23. The absorption spectrum of the isolated IgG in 0.1M phosphate buffer (pH 8.0) showed absorption maxima near 278 nm. Along with this, there was a minor hump at 290 nm and a trough near 250 nm. A fine structure near 258 nm was also observed. These structural characteristics of IgG molecule are indicative of the presence of phenylalanine, tyrosine and tryptophan amino acids. The spectral pattern of IgM showed a broad maxima in the region of 250 - 280 nm along with a trough near 240 nm. The obtained spectral characteristic of IgM is indicative of a higher phenylalanine content, which has caused a shift in the real maxima towards 250 nm. The absorption maxima for IgA was obtained at 276 nm.

F. MOLECULAR WEIGHT DETERMINATIONS BY SEPHADEX G-200

The molecular weight and the other hydrodynamic parameters of IgG were determined at pH 7.5 on a Sephadex G-200 column in 0.1M sodium phosphate buffer.

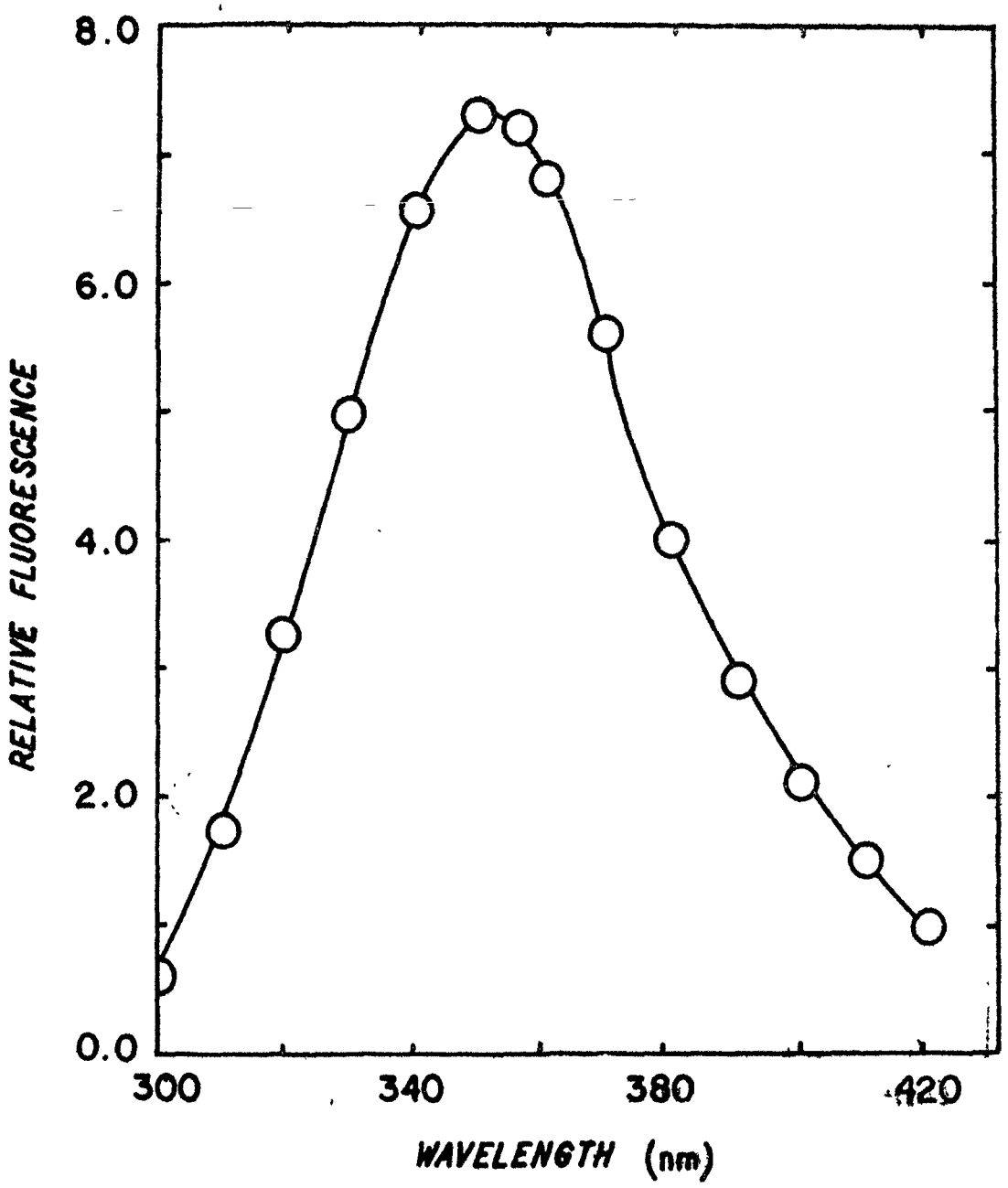


Figure 20. Emission fluorescence spectra of IgG in sodium phosphate buffer, 0.1M and pH 8.0. The spectra were recorded at 22°C in an Aminco-Bowman Spectrofluorometer using 0.5 mm slits. The fluorescence intensities were corrected for blank values before normalizing the emission spectra. The excitation wavelength was fixed at 280 nm.

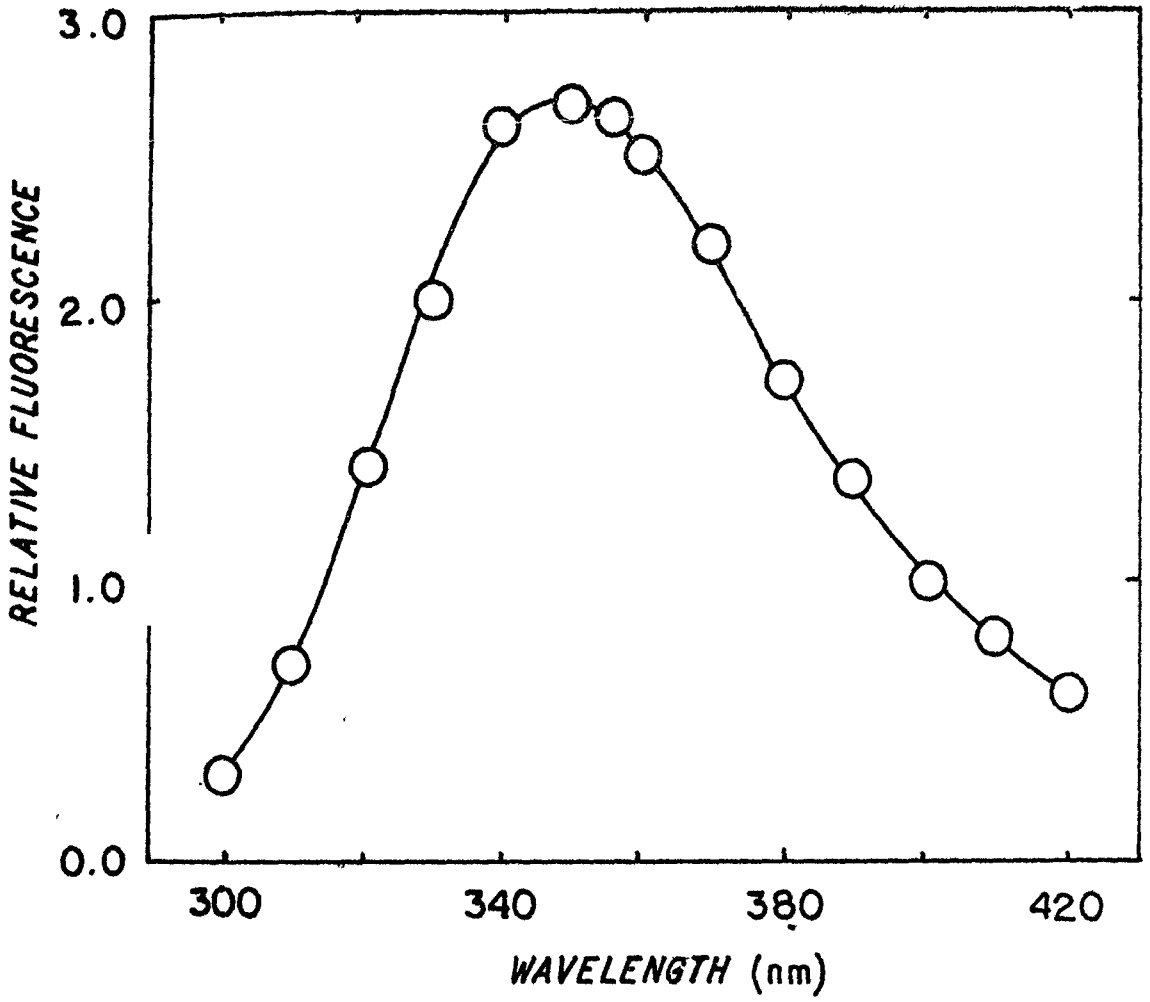


Figure 21. Emission fluorescence spectra of IgM in sodium phosphate buffer, 0.1M and pH 8.0. The spectra were recorded at 22°C in an Aminco-Bowman Spectrofluorometer using 0.5 mm slits. The fluorescence intensities were corrected for blank values before normalizing the emission spectra. The excitation wavelength was fixed at 280 nm.

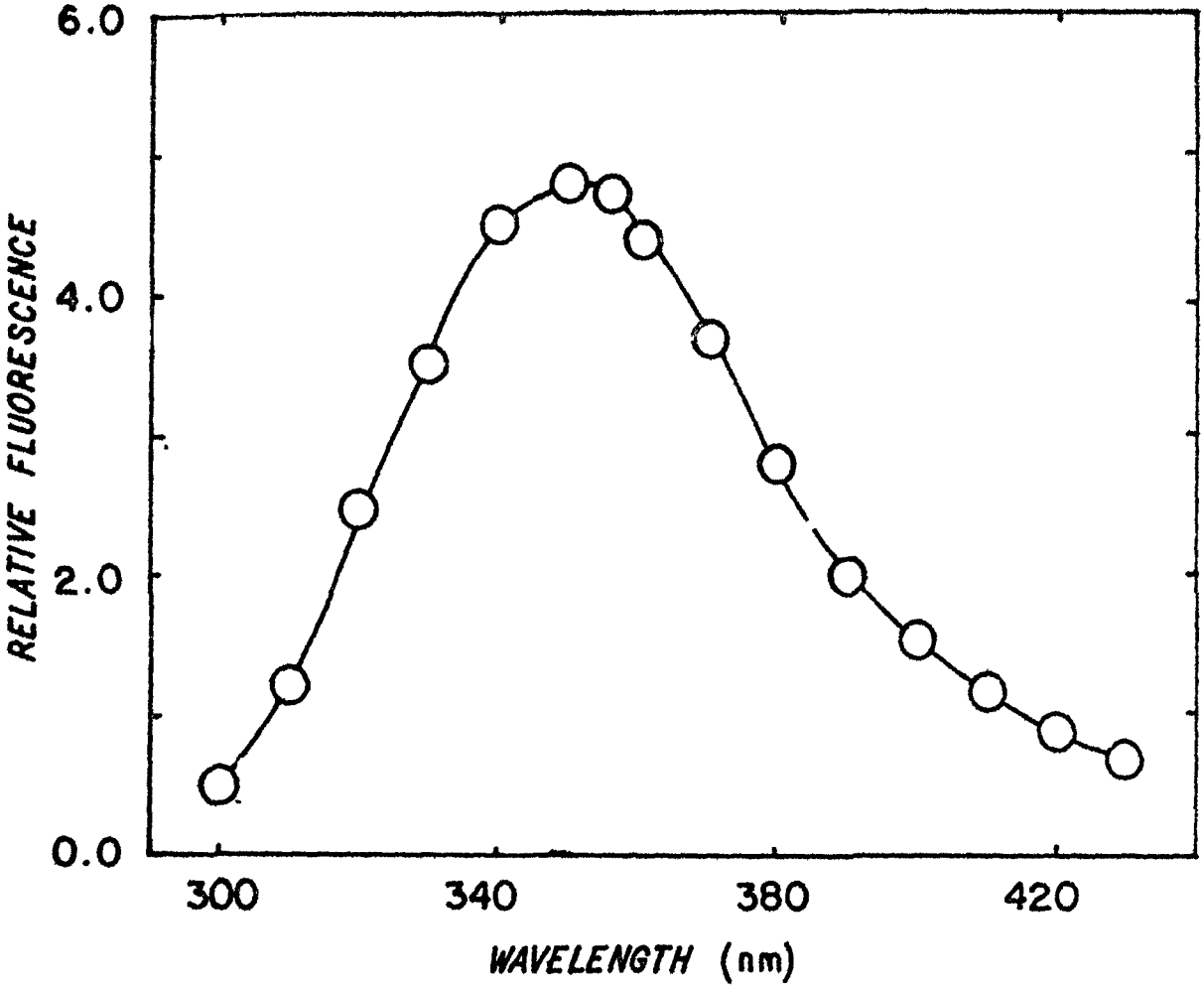


Figure 22. Emission fluorescence spectra of IgA in sodium phosphate buffer, 0.1M and pH 8.0. The spectra were recorded at 22°C in an Aminco-Bowman Spectrofluorometer using 0.5 mm slits. The fluorescence intensities were corrected for blank values before normalizing the emission spectra. The excitation wavelength was fixed at 280 nm.

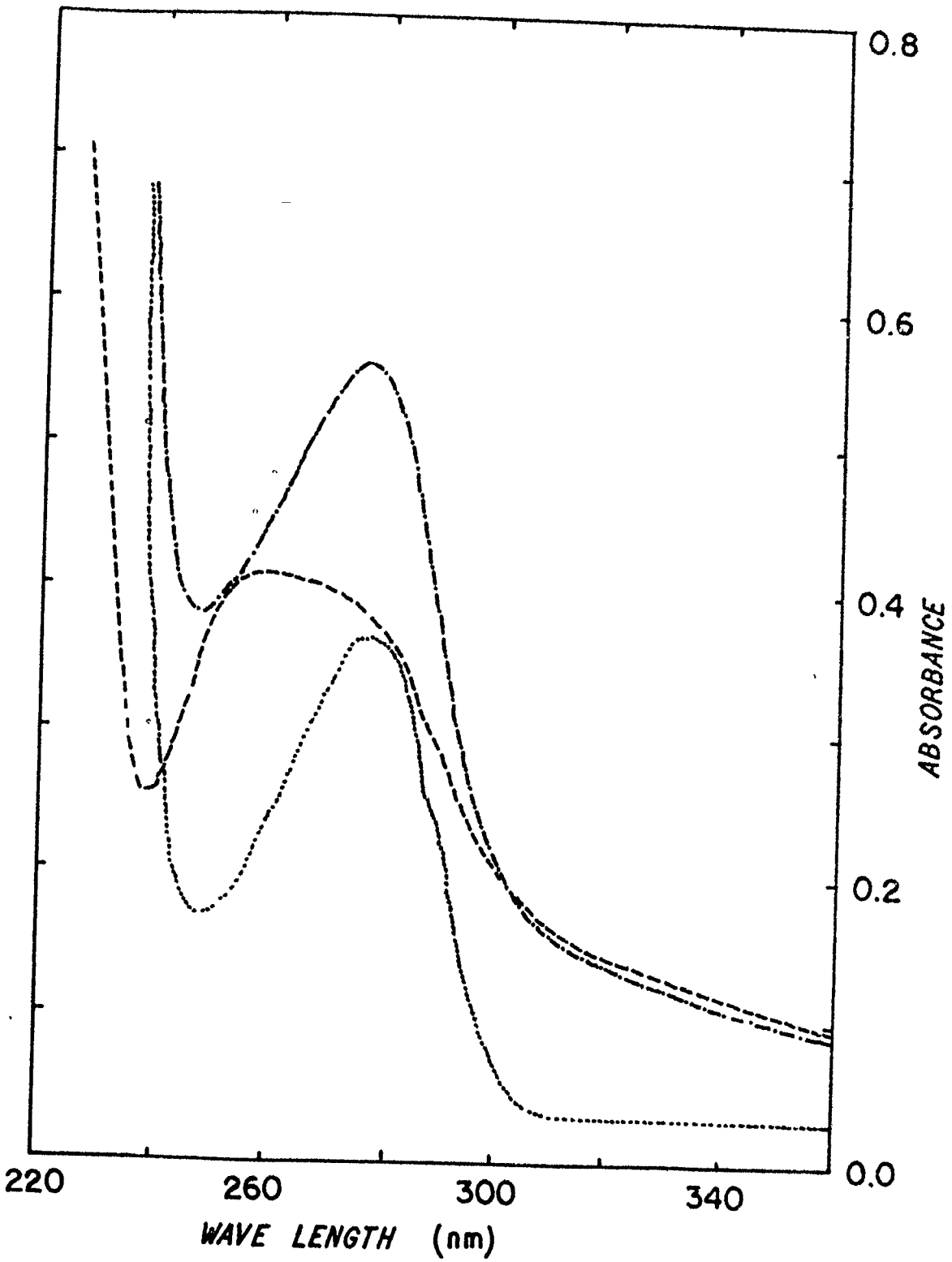


Figure 23. Ultraviolet absorption spectra of immunoglobulin G, M and A in sodium phosphate buffer, 0.1M and pH 8.0. The absorption spectra were recorded at 22°C in Beckman DK-2A ratio recorder spectrophotometer.
IgG (.....), IgM (-------) and IgA (-.-.-.-).

The gel filtration data were analyzed by employing the various parameters as follows: i) V_e/V_o - the ratio of the elution volume of protein to void volume, ii) K_d - the ratio of the elution coefficient and, iii) K_{av} - the available distribution coefficient. The column was equilibrated with 0.1M sodium phosphate buffer, pH 7.5. The test protein along with various marker proteins were eluted under identical experimental conditions. The elution profiles of the various proteins are shown in Figures 24 and 25. The elution volume was measured from the elution profile of proteins by extrapolating both sides of the peak to an apex, wherever needed. Blue dextran 2000 was used for the measurement of void volume which was found to be 72.5 ml. The void volume (V_o) of the colum was frequently checked during the course of these studies to measure the change, if any. However, no change in void volume was found during the experiment.

The marker proteins used in the experiment with their molecular weight and elution volumes are given in Table VII.

The following equations were used for the analysis of gel filtration data:

$$K_d = (V_e - V_o)/ V_i \quad \text{.....} \quad (1)$$

$$K_{av} = (V_e - V_o)/(V_t - V_o) \quad \text{.....} \quad (2)$$

Where K_d and K_{av} are distribution coefficient and available distribution coefficient, respectively. V_i is the volume of liquid inside

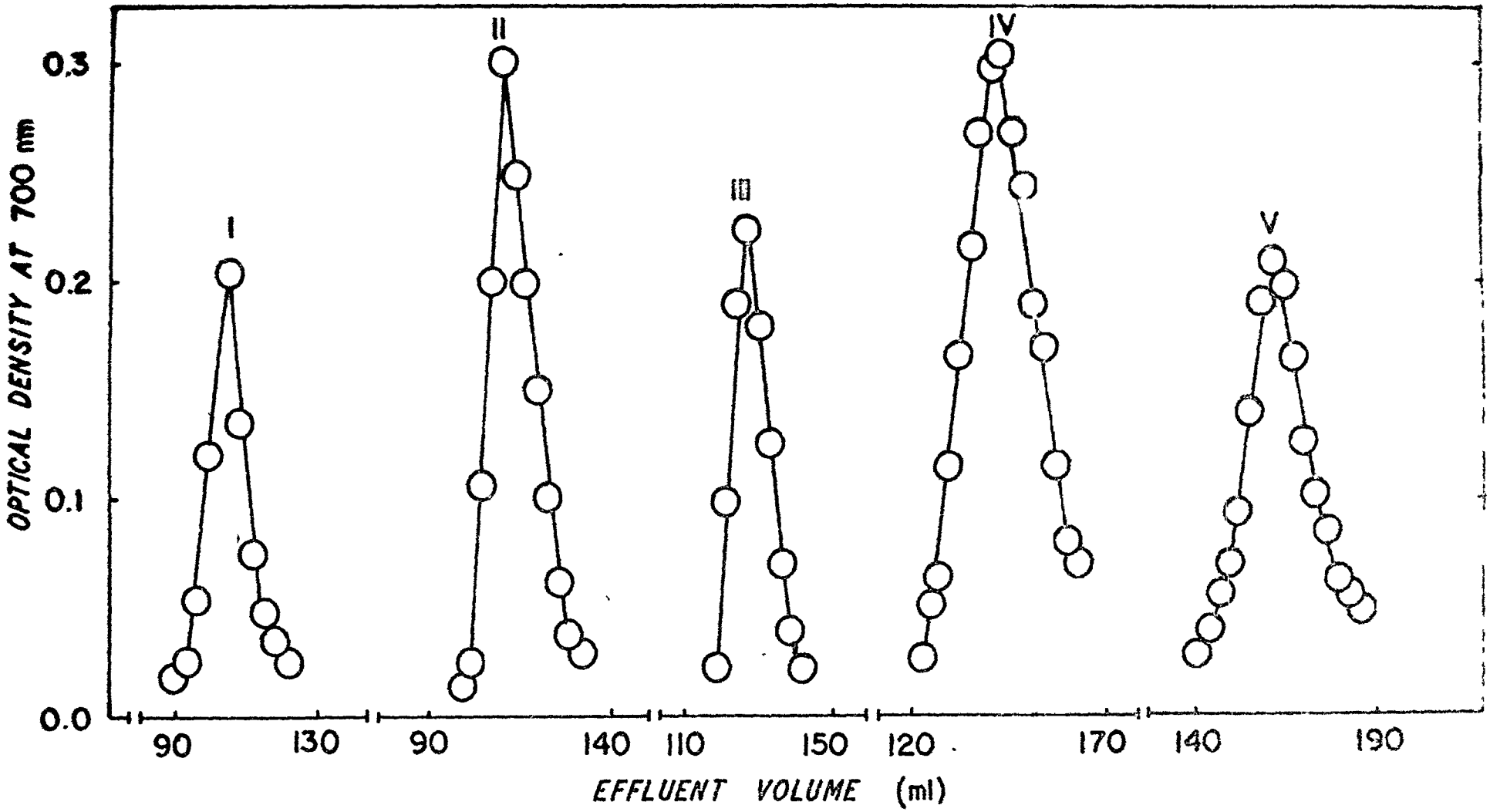


Figure 24. Gel filtration behaviour of marker proteins on Sephadex G-200 column.

Experimental conditions: About 4-8 mg of proteins were applied on to the column (2.1 x [illegible] cm) in sodium phosphate buffer 0.1M, pH 7.5 and eluted at a rate of 20 ml/hr. (i) IgG sheep, (ii) Bovine serum albumin (dimer), (iii) Bovine serum albumin (monomer), (iv) Ovalbumin and (v) α-Chymotrypsinogen A.

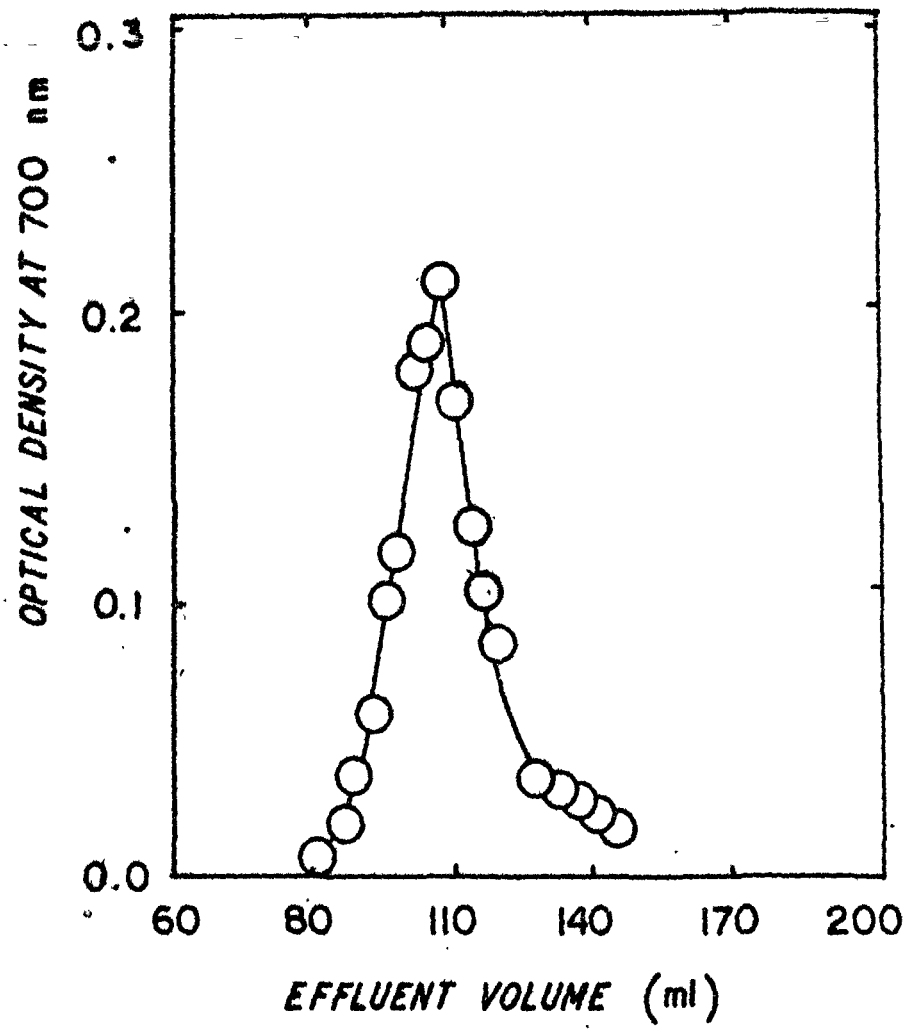


Figure 25. Chromatographic pattern of immunized rabbit IgG on Sephadex G-200.

Experimental conditions: A column measuring 2.1 x 54 cm was equilibrated with phosphate buffer of pH 7.5 and molarity 0.1. About 5.2 mg of protein was applied and eluted at a flow rate of 20 ml/hr.

TABLE - VII

Molecular Weight Parameters for Marker Proteins Used in Gel Filtration.

Protein	MW* X 10^{-4}	Stoke's Radius** (nm)	$V_e - V_o$ (ml)	V_e/V_o
1. α-Chymotrypsinogen A	2.57	2.24	89	2.24
2. Ovalbumin	4.30	2.73	73	2.01
3. BSA (monomer)	4.90**	3.55	55	1.76
4. BSA (dimer)	13.80**	4.30	38	1.53
5. IgG (Sheep)	16.00	4.84	34	1.47

* Values taken from Weber, K. and Osborn, M., J. Biol. Chem. 244: 4406 (1969).

** Values taken from Andrews, P., Methods in Biochemical Analysis 18: 1 (1970).

the gel material. The V_i was calculated from the reported value of gel matrix volume (V_m). V_t is the total volume of the gel bed, which was found to be 186.5 ml. The gel filtration data obtained for various marker proteins and IgG were analysed according to the theoretical treatment of Porath (180), Laurent and Killander (181) and Ackers (182).

According to the Porath, the distribution coefficient K_d of a protein molecule can be related to its Stoke's radius by the equation:

$$K_d = U (1 - r/ri)^3 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Where U is a constant. For rigid spherical molecules of similar density the Stoke's radius r_s is directly proportional to $M^{1/3}$. When $K_d^{1/3}$ was plotted against $M^{1/3}$ a linear relationship was observed as shown in Figure 26. The straight line was obtained using least square method and the equation of straight line as per below:

$$M^{1/3} = 118.5087 - 102.0953\ K_d^{1/3} \quad \ldots\ldots\ldots\ldots \quad (4)$$

Laurent and Killander (181) obtained an expression which shows the relationship between Stoke's radius and available coefficient, K_{av}. The expression predicts a linear relationship between $(-\log K_{av})^{\frac{1}{2}}$ and r, the Stoke's radius. Using the data of Table VIII, a plot of $(-\log K_{av})^{\frac{1}{2}}$ Vs Stoke's radius (r) was obtained by the method of least squares (Figure 27).

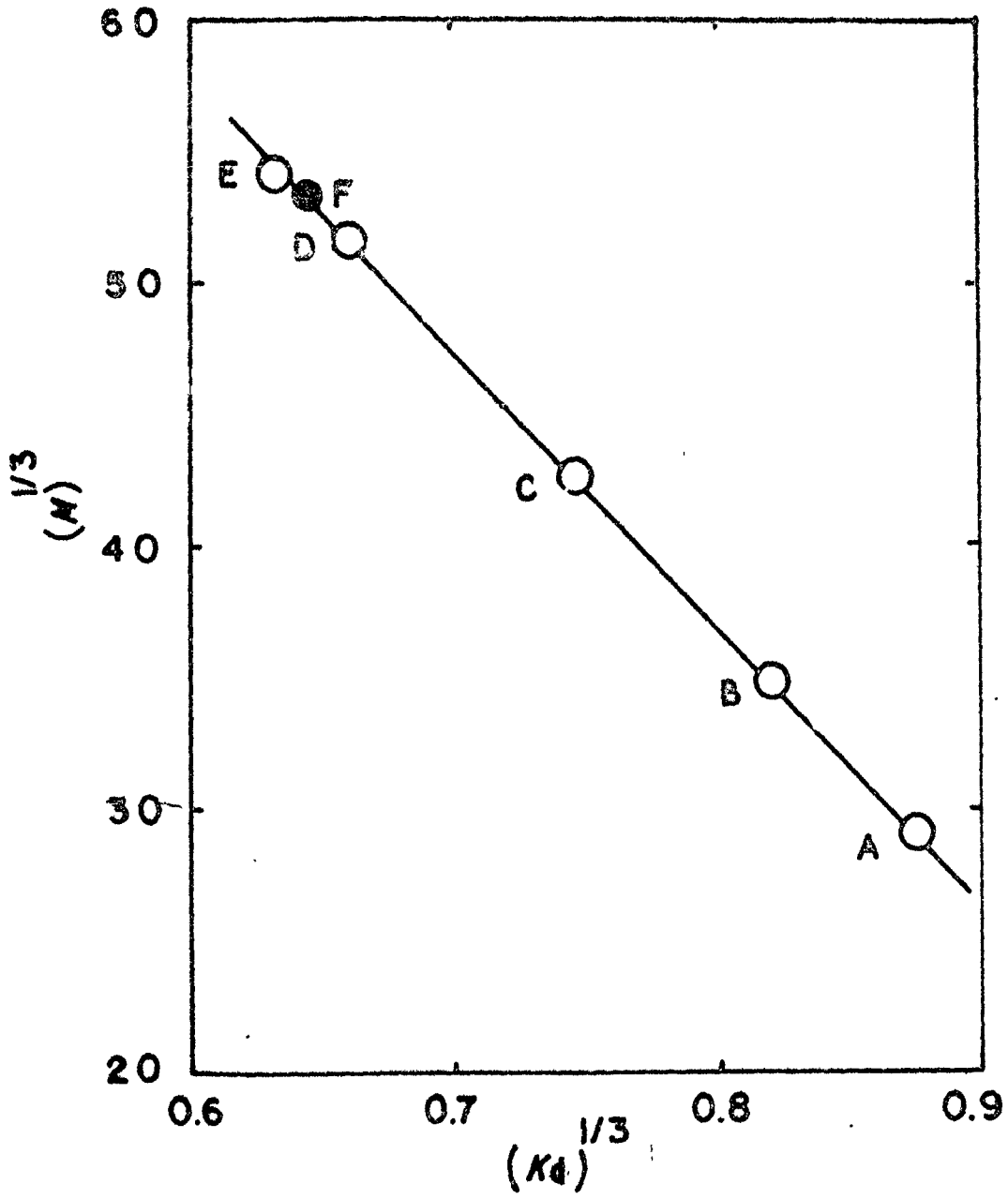


Figure 26. Plot of molecular weight Vs distribution coefficient according to the procedure of Porath (180). The straight line was drawn by the Least Square method (A, α-Chymotrypsinogen A; B, Ovalbumin; C, Bovine serum albumin (monomer); D, Bovine serum albumin (dimer); E, IgG (Sheep); F, antiameba IgG).

TABLE - VIII

Distribution Coefficient of Marker Proteins on Sephadex G-200 Column.

Protein	K_d	$K_d^{1/3}$	K_{av}	$(-\log K_{av})^{1/2}$
1. α -Chymotrypsinogen A	0.677	0.878	0.777	0.331
2. Ovalbumin	0.555	0.822	0.637	0.443
3. BSA (monomer)	0.481	0.747	0.480	0.564
4. BSA (dimer)	0.661	0.661	0.331	0.693
5. IgG (Sheep)	0.258	0.636	0.296	0.727

K_d : Distribution coefficient.

K_{av} : Available distribution coefficient.

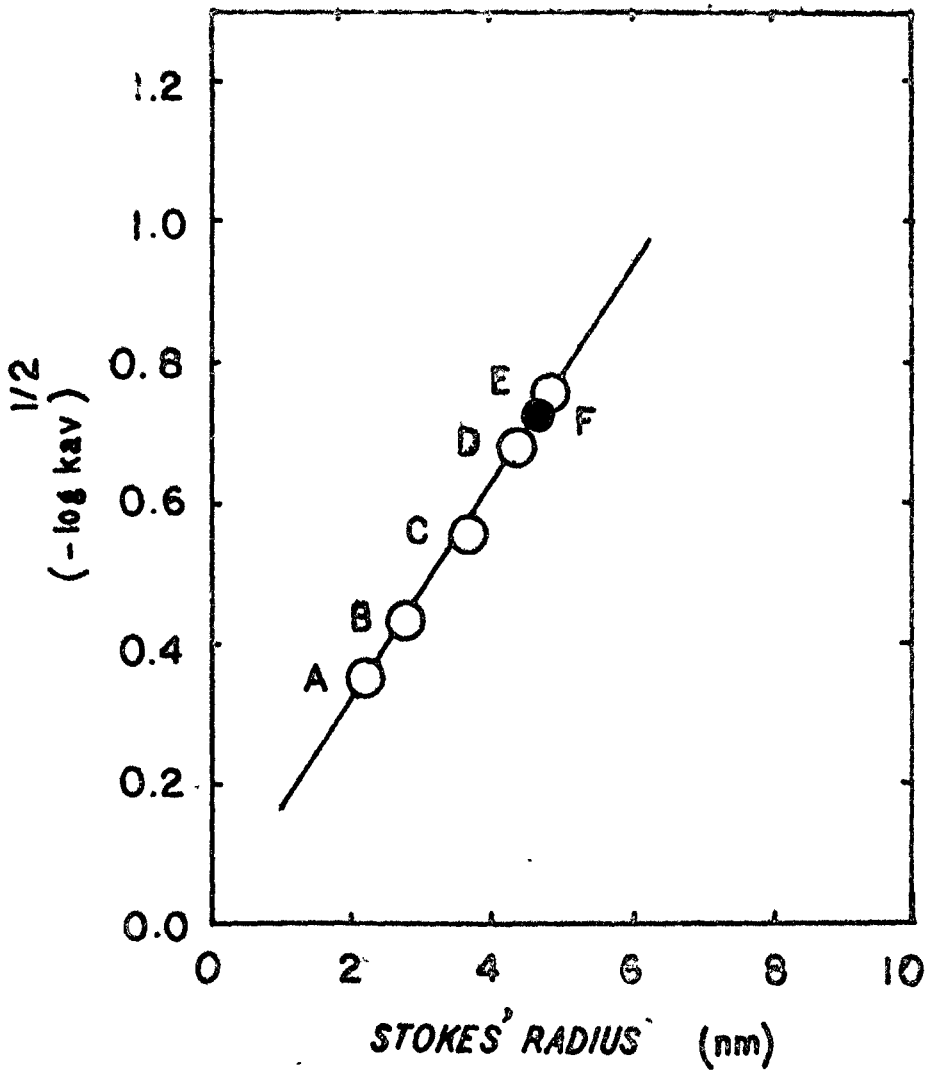


Figure 27. Plot of $(-\log Kav)^{\frac{1}{2}}$ Vs Stoke's radius of protein according to Laurent and Killander (181). The straight line was drawn by Least Square method (A, α-Chymotrypsinogen A; B, Ovalbumin; C, Bovine serum albumin (monomer); D, Bovine serum albumin (dimer); E, IgG (Sheep); F, antiameba IgG).

The experimental points fall on the straight line drawn by the method of least squares as per the following equation:

$$(-\log K_{av})^{\frac{1}{2}} = 0.156\ r - 0.00119 \quad \ldots\ldots\ldots\ldots\ldots \quad (5)$$

According to Ackers (102) the distribution coefficient K_d is given by the error function complement (erfc) of the Guassian distribution:

$$K_d = \text{erfc}\ (r - ro)/bo \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

$$r = ro + bo\ \text{erfc}^{-1}K_d \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

ro and bo are the calibration constants for the gel. Equation 7 shows a linear relationship between r and $\text{erfc}^{-1}K_d$ as given in Figure 28, which fits well into the following equation of straight line:

$$\text{erfc}^{-1}K_d = 0.199\ r - 0.132 \quad \ldots\ldots\ldots\ldots\ldots \quad (8)$$

The gel filtration behaviour of the proteins was found to be molecular weight dependent. The plot of V_e/V_o Vs log M for the marker proteins used in the experiment is shown in Figure 29. The relationship between log M and V_e/V_o was found to be linear for the molecular weight range which was employed in these investigations. The straight line was drawn by the least squares method and the equation of straight line is:

$$V_e/V_o = 6.413 - 0.994 \log M \quad \ldots\ldots\ldots\ldots\ldots \quad (9)$$

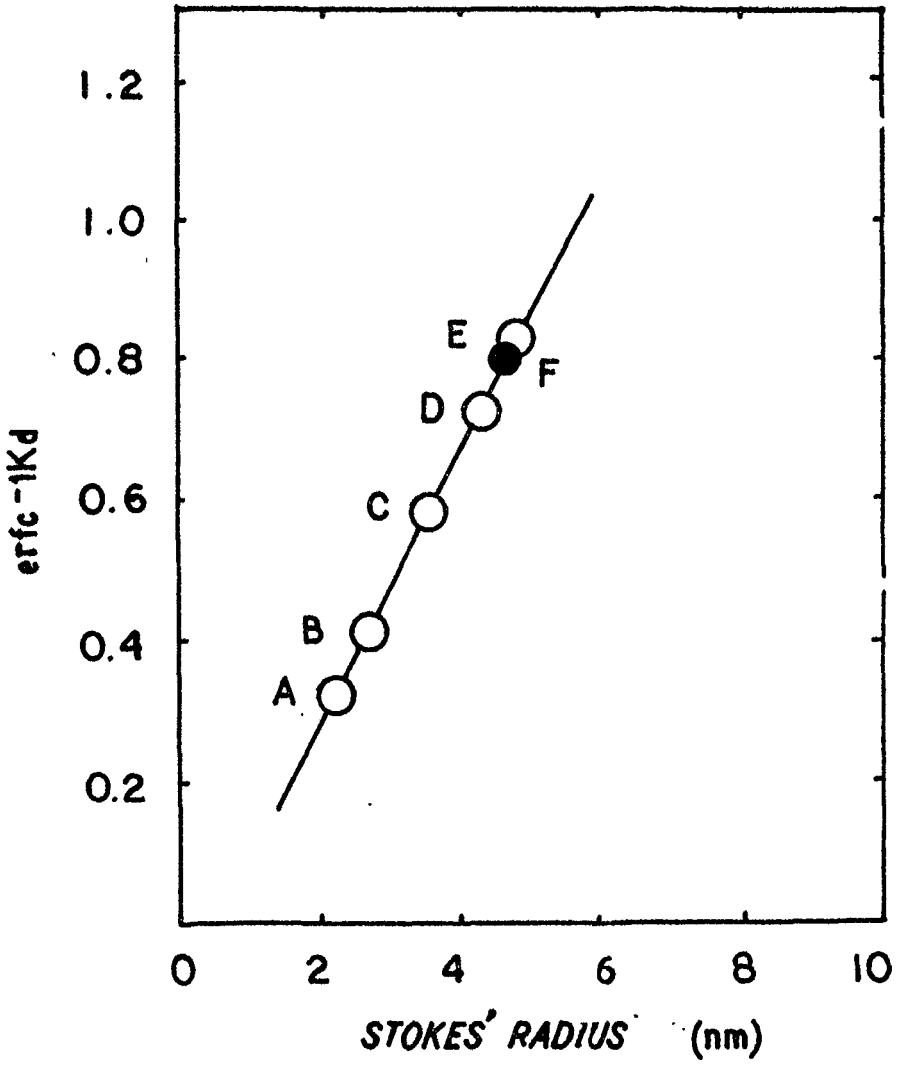


Figure 28. Treatment of gel filtration data of proteins according to Ackers (182). The straight line of the plot erfc^{-1} Kd Vs Stoke's radius was drawn by the method of Least Square. (A, α-Chymotrypsinogen A; B, Ovalbumin; C, Bovine serum albumin (monomer); D, Bovine serum albumin (dimer); E, IgG (Sheep); F, antiameba IgG).

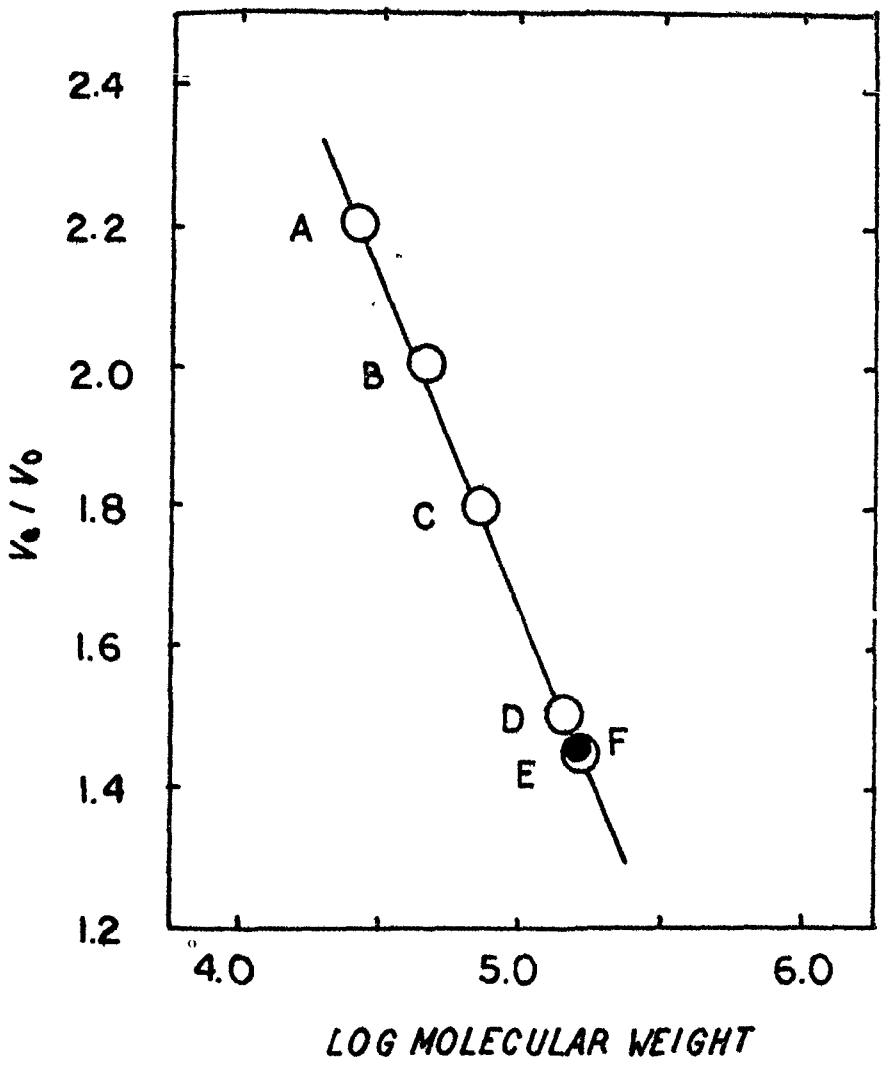


Figure 29. Plot of Ve/Vo Vs log M utilizing gel filtration data of proteins on Sephadex G-200 column. The straight line was drawn by Least Square method. (A, α-Chymotrypsinogen A; B, Ovalbumin; C, Bovine serum albumin (monomer); D, Bovine serum albumin (dimer); E, IgG (Sheep); F, antiameba IgG).

The various equations described above were used to calculate the molecular parameters such as Stoke's radius, r; distribution coefficient, K_d; and diffusion coefficient, D. The molecular weight of protein (IgG against E. histolytica) is given in Table IX. The values of ratio of elution volume to void volume, V_e/V_o; distribution coefficient, $M^{1/3}$; K_d and that of available coefficient for proteins at pH 7.5 on Sephadex G-200 column are detailed in Table X.

The molecular weight of IgG was calculated to be 149, 434 and 150,295 by the procedure of Andrews and Porath (180) respectively. Those values come out to be $149,865 \approx 150,000$. The Stoke's radii of IgG were found to be 4.60 nm, 4.60 nm and 4.64 nm by the procedures of Porath (180) Laurent and Killander (181) and Ackers (182) respectively. The average value comes out to be 4.613 ± 0.023 nm.

The diffusion coefficient, D, was calculated for immunoglobulin from Stoke's radius, r, using the following equation:

$$D = KT/6\pi\eta r \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

Where K is Boltzman constant (1.386×10^{-16} ergs/degree) η, is the viscosity of solvent in poise (0.010 poise) and T, is absolute temperature ($303^{\circ}C$). The diffusion coefficient of the portein molecule was calculated and found to be 4.83×10^{-7} cm^2/sec.

The frictional ratio, f/fo, was calculated from Stoke's radius by means of the equation:

TABLE - IX

Summary of Physico-Chemical Properties of Antiamoeba IgG.

Parameter	Determination	Values
Stoke's Radius	Porath (180)	4.60 nm
	Laurent & Killander (181)	4.60 nm
	Ackers (182)	4.64 nm
	Average	4.613 ± 0.023 nm
Diffusion Coefficient, D	From Stoke's Radius	4.83×10^{-7} cm^2/sec.
Molecular Weight	From Gel chromatography by Andrews procedure on Sephadex G-200 (175).	149,434
	From Porath plot (180)	150,295
		149,865 ± 608 ≈ 150,000
Frictional Ratio, f/fo	From Stoke's radius	1.318

TABLE - X

Gel Filtration Data of Antiamoeba IgG and Standard Proteins.

Proteins	Stoke's radius (nm)	V_e/V_o	K_d	$(K_d)^{1/3}$	K_{av}	$(-\log K_{av})^{\frac{1}{2}}$	$erfc^{-1}K_d$
α-Chymotrypsinogen A	2.24	2.22	0.672	0.876	0.776	0.332	0.2998
Ovalbumin	2.73	2.00	0.551	0.820	0.636	0.443	0.4228
Bovine Serum Albumin (monomer)	3.55	1.75	0.414	0.745	0.478	0.556	0.5798
Bovine Serum Albumin (dimer)	4.30	1.52	0.285	0.658	0.329	0.695	0.75566
IgG (Sheep)	4.84	1.46	0.254	0.633	0.294	0.729	0.8075
Antiamoeba IgG	-	1.47	0.262	0.640	0.303	0.720	0.7947

V_e/V_o : Ratio of elution volume to void volume.
K_d : Distribution coefficient.
K_{av} : Available distribution coefficient.
erfc : Error function complement.

$$f/fo = r/(3\,\bar{V}_2\,M/4\,\pi\,N)^{1/3} \quad \ldots\ldots\ldots\ldots\ldots \quad (11)$$

Where $\bar{V}_2$ is the partial specific volume of the protein and N, the Avagadro's number. A value of 0.72, ml/gm of $\bar{V}_2$ for Bovine IgG (183) was used. The fractional ratio of the IgG was determined as 1.318. All the above values are shown in Table IX.

III. CELL-MEDIATED IMMUNE RESPONSE

A. SKIN TEST

The results of skin hypersensitivity tests show that all the guinea pigs from the immunized groups developed a positive hypersensitivity skin reaction. The control animals did not show the development of any skin reaction. A positive skin reaction was characterized by erythema and induration which appeared only after several hrs. Such positive reactions reached a maximum at 24 - 48 hrs and subsided thereafter. The diameter of the skin reactions developed by using 8.8 ug and 4.4 ug of antigen were 15 mm and 10 mm, respectively. The above findings were obtainable after 24 hrs in almost all the antigen-immunized groups. After 48 hrs, the reaction in groups III and IV (receiving 70.50 and 141.00 ug of antigen) were somewhat weak. In group II (receiving 35.25 ug of antigen), the skin response became very weak; the diameter of the reaction was, infact, less than 5 mm. The details of delayed hypersensitivity reactions are represented in Figure 30 and Table XI. The diameter of the skin reaction measuring less than 5 mm was considered as negative.

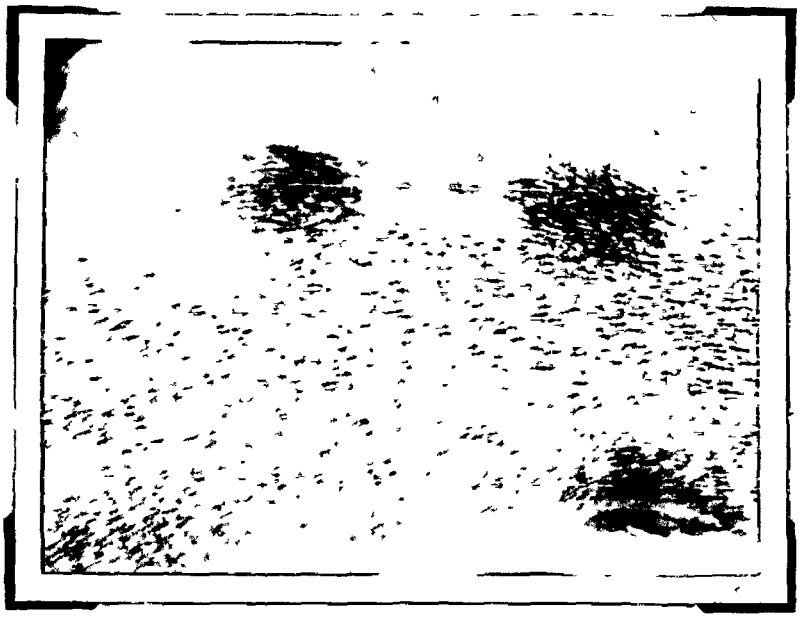


Figure 30. Delayed hypersensitivity reaction in guinea pig against E. histolytica antigen.

TABLE - XI

Details of Skin Hypersensitivity Reaction in Guinea Pigs

Groups of guinea pigs	Total Antigen used for immunisation. in ug	Antigen used in Skin Test		Diameter of Skin Test after 24 hrs	
		8.8 ug	4.4 ug	(8.8 ug antigen) Skin reaction in mm	(4.4 ug antigen) Skin reaction in mm
Unsensitised (fresh)	Control	ND	ND	No reaction	No reaction
I	Saline	8.8	4.4	No reaction	No reaction
II	35.25	8.8	4.4	15.00	10.00
III	70.50	8.8	4.4	15.00	10.00
IV	141.00	8.8	4.4	15.00	10.00

ND = Not done.

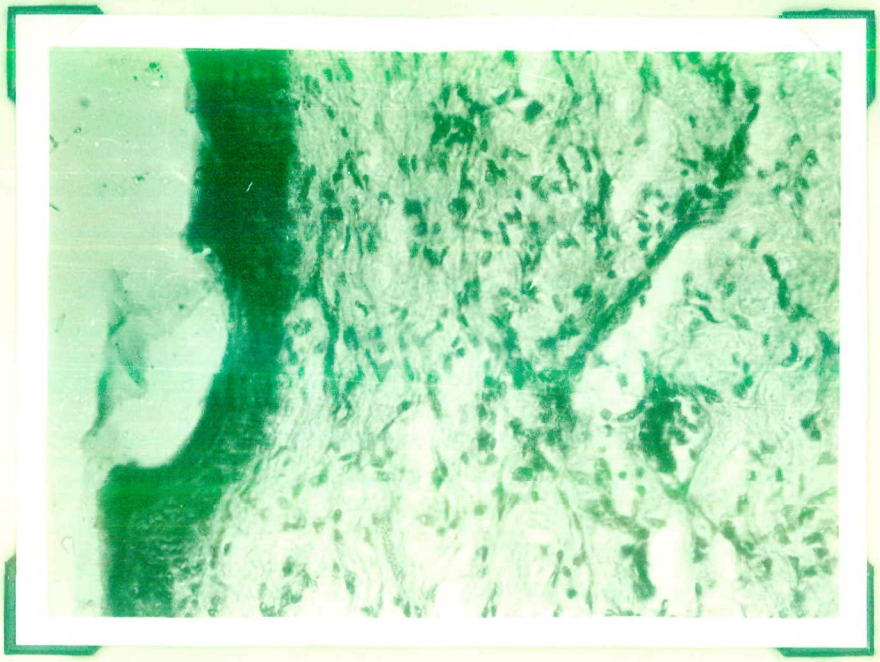
B. HISTOLOGICAL STUDY OF THE REACTION SITES

Lesions caused by inoculating soluble antigen were surgically removed from guinea pigs and fixed in 10 per cent sodium phosphate-buffered formalin. Subsequently, the tissues were prepared for microscopic examinations. The lesion sites were characterized by perivascular cuffing and diffuse infiltration of the intra-cellular spaces by mononuclear cell-types (lymphocytes and macrophages). A few polymorphonuclear leukocytes were also observed as seen in Figure 31.

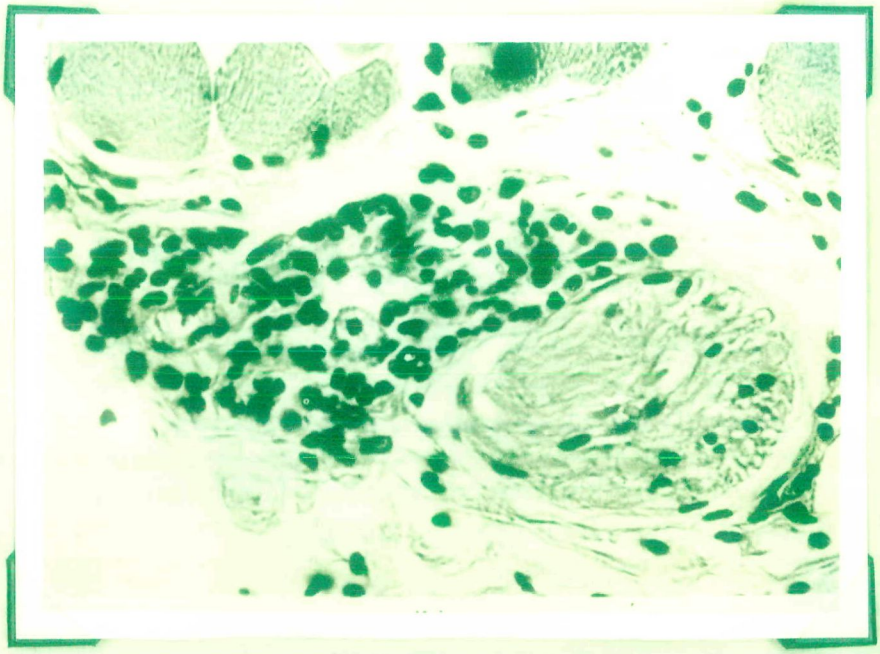
C. IMMUNOLOGICAL INVESTIGATIONS

1. Migration Inhibition by Capillary Tube Method

The migration of peritoneal cells from animals sensitized with _A. histolytica_ antigen was markedly inhibited by 0.38 ug of antigen. These observations were made about 12 hrs after the test. The comparison of the test and control tubes shown in Figure 32. The results of migration inhibition are represented in Table XII. On the basis of these results, it can be observed that cells from the sensitive animals were significantly inhibited, in comparison to those from normal animals. The quantitative inhibitory effect of antigen on the migration of sensitive cells appears to be quite significant. On the other hand, the control cells migrated out of the capillary tubes in a lacy fan-like pattern. The inhibited cells were prepared as a dense clump with a smooth, clearly defined border.



CONTROL (A)



TEST (B)

Figure 31. Photomicrographs from cutaneous delayed hypersensitivity reaction sites. A. Saline injected control site. B. Delayed hypersensitivity reaction site. The infiltration of mononuclear cells with some leukocytes can be seen in the photograph.

TABLE - XII

Details of Migration Inhibition in Capillary Tubes.

Groups of guinea pigs	Antigen used for immunization in ug	Antigen used in migration inhibition in ug	% migration inhibition (± SD)	Significance
Fresh	ND	0.88	0.00	-
I	Saline	0.88	5.19 ± 2.97	-
II	35.25	0.88	31.53 ± 0.35	P < 0.01
III	70.50	0.88	48.92 ± 2.87	P < 0.01
IV	141.00	0.88	44.91 ± 7.78	P < 0.01

ND = Not done;
Significance of difference between various groups was calculated on the basis of student 't' test.

Apparently, there appears to be no direct correlation with inhibition of cell migration and area diameters of skin reaction sites. In order to correlate the appearance of cell-mediated immunity during infection, it was found that 1:4 (0.88 ug antigen) dilution is the optimal dilution for studying the migration inhibition and skin reaction tests. A 1:2 (1.716 ug antigen) dilution, when used in these tests, was found to give a lesser degree of migration inhibition than the one obtained from 1:4 (0.88 ug antigen) dilution. The results of these experiments showed that the third dilution (70.5 ug antigen) was the optimal dilution and apparently appeared to be more immunogenic for elicitating cell-mediated immune responses.

2. Migration Inhibition by Agarose Method

The results of migration inhibition are presented in Table XIII. The lowest values were recorded from animals immunized with lower doses of antigens. The lower value (3 per cent) inhibition was obtained in the controls as well as from the first antigen dilution (35.25 ug antigen). These values, however, are not statistically significant ($P > 0.2$). In III and IV groups of animals receiving (70.5 ug and 141.00 ug antigen), the migration inhibition values were 7.92 ± 15.2 and 9.8 ± 5.7 per cent respectively - indicating a specific inhibition by ameba antigen. These values are statistically significant with respect to controls. During inhibition, only two antigen dilutions were used (i.e. 1:2 and 1:4 containing 1.76 and 0.88 ug antigen). Dilution 1:4

TABLE - XIII

Details of Migration Inhibition in Agarose Plates.

Groups of guinea pigs	Antigen used for immunization in ug	Antigen used in migration inhibition in ug	% migration inhibition (± SD)	Significance
Unsensitized (Fresh)	ND	0.88	0.00	-
I	Saline	0.88	3.00 ± 10.6	NS
II	35.25	0.88	3.00 ± 00.0	NS
III	70.50	0.88	7.92 ± 15.2	P < 0.01
IV	141.00	0.88	9.80 ± 5.7	P < 0.01

ND = Not done; NS = Not significant.

Significance of difference between groups was calculated on the basis of student 't' test.

TABLE - XIII

Details of Migration Inhibition in Agarose Plates.

Groups of guinea pigs	Antigen used for immunisation in ug	Antigen used in migration inhibition in ug	% migration inhibition (± SD)	Significance
Unsensitized (Fresh)	ND	0.83	0.00	-
I	Saline	0.88	3.00 ± 10.6	NS
II	35.25	0.88	3.00 ± 00.0	NS
III	70.50	0.88	7.92 ± 15.2	P < 0.01
IV	141.00	0.88	9.80 ± 5.7	P < 0.01

ND = Not done; NS = Not significant.

Significance of difference between groups was calculated on the basis of student 't' test.

(0.38 ug antigen) was found as the optimal dilution for the inhibition of sensitized peritoneal exudate cells in agarose plate (Figure 33). The results in the above two tests are somewhat contradictory due to the differences in the respective technics, and also due to the varied specificity of the tests. The agarose plate technic is very specific for purified antigen, while the capillary tube technic is specific for crude type of antigens only. The amoba antigens used in these tests were crude type and therefore more appropriate for being employed in capillary tube tests. The values obtained in capillary tube tests are slightly higher than those obtained from the agarose plate method.

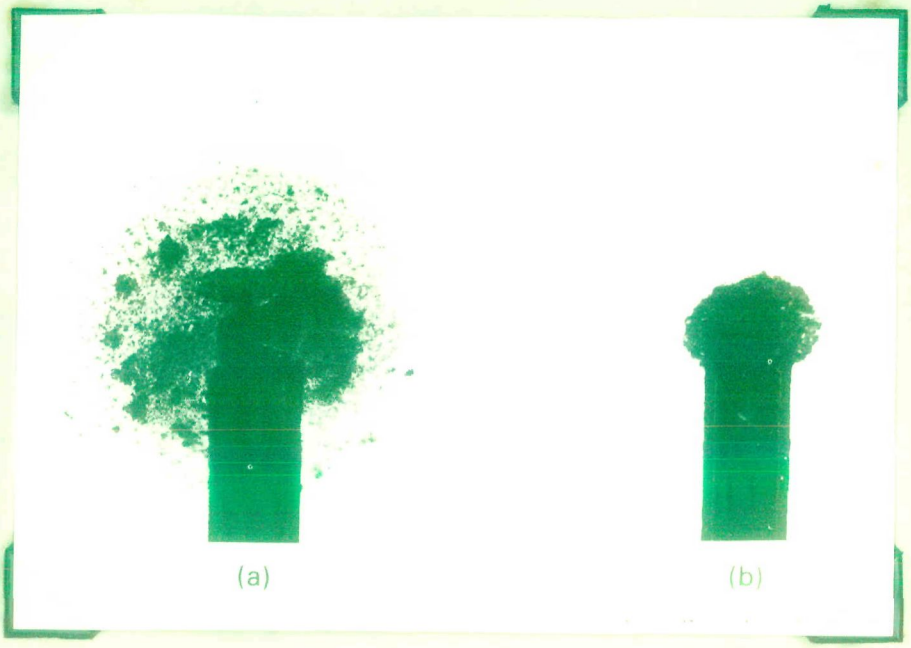


Figure 32. Inhibition of E. histolytica antigen-sensitized guinea pig peritoneal cells in the presence of homologous antigen. (a) Migration in control culture without antigen, (b) Inhibition of migration in the presence of antigen in capillary tube tests.

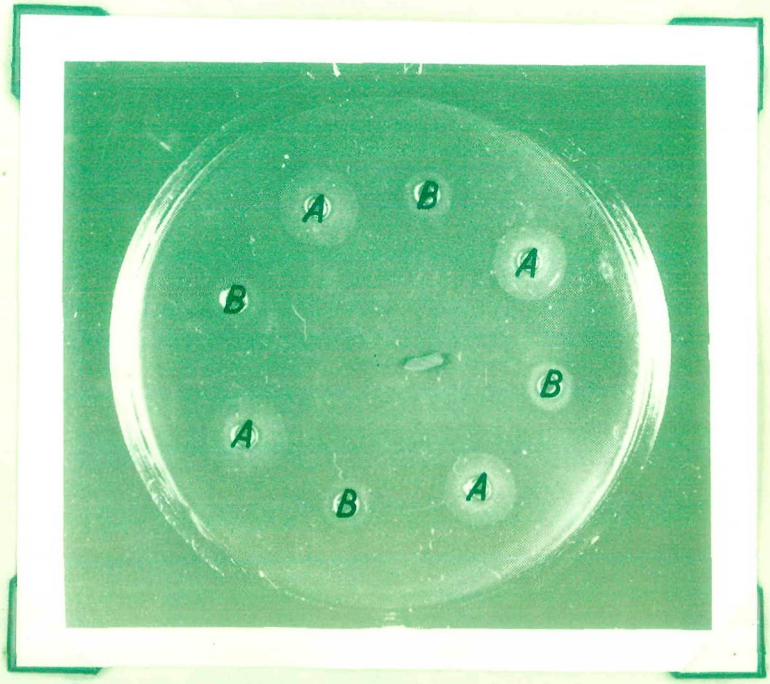


Figure 33. Inhibition of E. histolytica antigen-sensitized guinea pig cells in the presence of homologous antigen. (a) Migration in control culture without antigen; (b) Inhibition of migration in the presence of antigen in agarose plate tests.

CHAPTER - V

D I S C U S S I O N

The appearance of immunity against several diseases of parasitic origin is attributed to the formation of specific serum proteins or antibodies. These antibodies are produced by vertebrates in response to foreign invaders or antigens. Immune response, or the production of antibodies in general, depend upon the makeup and the purity of antigens. The axenic cultivation of E. histolytica by Diamond (18), for the first time, made it possible to prepare pure ameba antigen extracts. The axenic technic provided an opportunity to investigate the specificity of antigens by enhancing the sensitivity and the reactivity of pure antigen preparations. This also provided an opportunity to investigate the exact immunological responses obtainable during an amebic infection. The antigenic extract used in the present study was made from axenic culture of E. histolytica prepared according to the method of Kessel et al. (111).

The water soluble fraction of E. histolytica was employed to test the elicited immune responses in experimental animals and, also in amebic patients. These antigens were used to assess the humoral and cellular immune responses. The soluble antigen preparation was quite capable of eliciting a humoral antibody response in experimental animals. During the humoral immune response, different manifestations of antibody activity were generally obtainable. Antibody activities were detectable as precipitin, hemagglutinin, complement fixing and skin reactive activities. The IHA test was found as the most sensitive for the detection of antibodies during parasitic infection. This was then

followed by complement fixation, precipitin tests, fluorescent antibody tests and skin hypersensitivity tests (86).

The aim of the present investigation was to characterize the amebic immune sera against E. histolytica antigens. The normal and immunized rabbit sera samples resolved into three well defined peaks on DEAE-cellulose (IgG, IgA and IgM) respectively, in the presence of a salt gradient. These results are in conformity with the findings of Yap et al. (90). In the absence of any salt gradient, only one class of immunoglobulin was resolvable, giving rise to only one peak. Due to differences in the ionic charges on immunoglobulin molecules belonging to respective classes, the employment of a salt gradient was found necessary for purifying the molecules. The immunoglobulins were separated on DEAE-cellulose by eluting from the ion exchange matrix with buffers of different ionic strengths. Immunoglobulin G, A and M were separated from the DEAE-cellulose column at 0.0175, 0.035 and 0.15 molar phosphate buffers respectively. The results of these separations are in correspondence with those of Maddison (184). Fractionation of antiamebic sera by Sephadex G-200 also gave rise to three peaks. Peak I and II correspond to IgM and IgG respectively as according to Yap et al. (90). The third peak obtained from Sephadex G-200 mostly contained a mixture of immunoglobulins and albumin. IgA was not quite well resolvable, separately. The two immunoglobulins (IgA and IgG) appeared to have eluted simultaneously because of their identical molecular weights. Yap et al. (90) have also described similar findings in their investigations in which IgG

was eluted in peak I on DEAE-cellulose column, and in peak II on Sephadex G-200 column.

The fractions obtained from DEAE-cellulose columns were checked on Sephadex G-200 column for heterogeneity on the basis of their molecular size. The fractions obtained from DEAE-cellulose were not found homogeneous on the basis of molecular size. An apparently homogeneous peak obtained on Sephadex G-200 was also found to possess microheterogeneity in polyacrylamide gel electrophoresis. But these peaks corresponded well with the standard immunoglobulins which were used against them. In sequential sera samples, both the IgM and IgG antibodies appeared simultaneously during the primary response, as a result of active immunization with E. histolytica antigens. This was confirmed by carrying out serum electrophoresis in agar. The sequential appearance of antibodies was in decreasing order from first to third week as depicted in Figure 13b. As a result of a booster injection in the 4th week, the antibody level was considerably increased from 3rd week to 5th week.

In primary response sequential sera, both the IgM and IgG were present but the hemagglutinin levels were quite low (Figure 16). Whereas in secondary response sera, the hemagglutinin and precipitin levels were considerably increased. The level of IgM increased considerably from 2 mg in 3rd week to 4.95 mg in the 5th week. The IgG level in the corresponding period increased from 13.77 mg to 14.96 mg. Immunoglobulin G was found to be the most reactive

antiamebic immunoglobulin, although the hemagglutinin activity was present in both IgM and IgG classes of antibodies. Subsequently, the level of antiamebic antibodies increased greatly and remained so until 12th week.

Abioye *et al.* (91) has stated that of all the three immunoglobulins (G, A and M), IgG is the one which is mainly involved in amebiasis, and is also generally found elevated during an active disease state. Production of IgG class of antibodies may therefore be regarded as one of the most important immune mechanisms which is elicited by the host in human amebiasis. Such antibodies appear in significant amount in response to a specific stimulus, and react well with the specific antigen-*E. histolytica*, or its fractions. This study further confirms the findings (Boonpucknavig and Bhairn (93), Chiddison *et al.* (86), and Tavanat and Chaicumpa (92)) that the antibody activity in amebiasis is mainly associated with IgG fraction of the serum. The involvement of IgG as the main antibody is further supported by the fact that there is a progressive decline of the IgG level during treatment. This is highly suggestive of the fact that the specific activity and the raised level of this type of immunoglobulin is produced in response to host's immune defense against an amebic infection. The results of the present studies are in agreement with the findings of Ali Khan and Meerovitch (84), where IgG antibodies increased considerably with booster injections of ameba antigen given during the immunisation of rabbits. It is, however, not certain whether the IgG antibodies are protective against reinfection, or not. As far as reinfection is concerned,

it is quite possible that IgG antibody response is only transitory with a rapid decline subsequently in the specific antibody level during convalescence - exposing the individual to the risk of reinfection.

The role of IgA antibody in the mechanism of immunity is not clearly understood. In patients of liver abscess, the IgA were found elevated. However, those levels are not elevated as far as that of IgG (185). It is also reported that during infections of cholera and poliomyelitis the IgA antibody play an important role of protection (88,89). Cepulveda (186) has also detected the presence of IgA at the surface of rectal mucosa in patients with invasive amebiasis. It is also believed that there is a local protective activity of the secretory IgA against an amebic infection. In the present investigations, the IgE antibody was not isolated and purified from the immunized sera. Ishizaka et al. (187) have isolated and purified the IgE-type homocytotropic antibody from rabbit against DNP-BCG with Freund's complete adjuvant. They have also reported that the antibody was found to appear six days after the primary inoculation and, disappeared in two to three weeks. The antibody always appeared in the same sequence after giving secondary antigenic stimulations. The antibody was also found to be inactivated by mercaptoethanol, reduction and alkylation. Dobson et al. (188) have also demonstrated a guinea pig IgE reaginic antibody against Ascaris suum infections. These studies have shown that in addition to 7S IgG1 and 7S IgG2 group of antibodies, another anaphylactic antibody analogous to human IgE is also present. This

was established by taking into consideration such factors like molecular weight, 2-mercaptoethanol sensitivity, heat liability, and ability to sensitise homologous skin sites. Ogilvie and Jones (189) have also described the formation of reaginic antibody as a general phenomenon in helminth infected animals. From these studies it can be concluded that PCA activity of the whole antiserum is mediated by either IgG or IgE, or, simultaneously by both classes of antibodies (190-193).

Bhagupta (194) has also reported high levels of IgE in patients of intestinal amebiasis. High level of IgE was also demonstrated in patients with bronchial asthma of various grades in normal subjects with and/without parasites in stools. Generally, it was observed that there is a tendency of increasing IgE concentrations in those who harbour parasites or suffer from subclinical infections with parasites. The association between cell-mediated and IgE-mediated immunity to pollen antigen has also been demonstrated. The T cell has recently been shown to have a role in helping B cell for the production of reaginic type of antibody (195,196).

The participation of both IgG and IgE-type of antibodies was found in PCA reactivity of anti-*E. histolytica* guinea pig serum (197). These investigations indicate that besides IgG, the IgE-type reaginic antibody also participates in these reactions during the peak primary and secondary humoral antibody responses in guinea pigs, which were previously immunised with *E. histolytica*

antigen. Skin histological studies of the peak cutaneous anaphylactic reactions also indicated the participation of IgE-type antibody. An accumulation of PMN leukocytes (predominantly eosinophils) at the reaction sites was also observed.

The detection of antiamebic antibodies in the present investigations was carried out by using precipitin, indirect hemagglutination, immunodiffusion, immunoelectrophoresis, fluorescent antibody, complement fixation, bentonite slide flocculation and skin hypersensitivity tests. The results are more or less in perfect agreement with a large number of earlier investigations of a similar nature. Among these tests, the IHA is the more reliable and sensitive test procedure. The test is also specific for the detection of antiamebic antibodies from experimental animals and is equally efficient for the purpose of diagnosis of amebiasis in patients. Hilgram et al. (107), Kessel et al. (113), Sherovitch and Ali Khan (198) and Healy (106) have all reported similar results which were obtained from their studies. The titres obtained in the present studies were much higher (1:4096) than reported earlier. Krupp and Powell (96,114) have carried out extensive studies on antibody response to invasive amebiasis in Durban, South Africa. They have found that immunodiffusion and indirect hemagglutination tests are almost similar in their ability to detect antibodies. Positive results upto the extent of 95 per cent were routinely obtainable by the above as well as by various other workers (199,200). The few discrepancies often noticeable in the results were almost exclusively reported from patients with

low antibody titres of 1:243 or less. Since, over 10 per cent of patients from amebic dysentery and liver abscess groups show IHA titre only of a relatively lower order. But a definite correlation between the antibody level and the severity of infection, or the extent of tissue invasion, cannot be reliably established on the basis of IHA titres only. Although, Krupp (199) has shown that there is a greater antibody production in cases of severe tissue invasion. But such finding is not always applicable because of considerable individual differences in the elicitation of immune responses to amebic invasion. In spite of good immune responses (high level of IHA titre) in several cases, it has been noted that amebic antibodies were not able to protect the individual against reinfection. But at the same time, the frequency of reinfection in a large number of patients in some way remained quite low, upto a period of six months.

In the present study only 5 precipitin bands were generally available, while Ali Khan and Meerovitch (84) have reported the appearance of 7 precipitin bands in their immunodiffusion tests. This difference may have only crept in due to the type of antigen preparation used in the respective tests. Such minor differences could also be explained on the basis of immune behaviour of the experimental animals. But one thing is certain that E. histolytica comprises of multiple antigenic sites and that, antibody is synthesised against all the antigenic sites present on the amoeba. The ID test is slightly less sensitive than the IHA test. Further, it has also been observed that whenever, the IHA titres are found around

1:256 or more, the gel test appears to be automatically positive. Sherovitch and Ali Khan (198) have also made similar observations in which the gel test was found positive when the IHA titres were recorded as 1:512 or more. The gel test is generally considered better for the purpose of making clinical diagnosis, as it gives less false positives compared to the IHA test (201). The ID test also appears to be more discriminatory, as the results correlate better with the clinical findings. Because of high sensitivity of the gel test, it is a good procedure for clinical screening of amebic infections. The gel test is also useful in assessing the clinical significance of a positive IHA test. The results of ID and IEP studies on crude extracts and its various fractions show, a more or less, similar distribution of precipitating antigen. The precipitin bands obtained in the present study do not correlate well with the previous work of a similar nature (91,202). Although the results of bentonite slide flocculation test are in perfect agreement with the work of Tupasi and Healy (124). The BTF test was found highly specific. No cross-reactions were observed when the test was evaluated against sera from normal controls and patients having gastrointestinal complaints, or having parasitic, bacterial and viral infections. A positive BTF test is a reliable indication of an active or past amebic infection.

The results of immunofluorescence tests are also in agreement with the results of a large number of other studies. Boonpucknavig and Saira (93) have described that 85 per cent of cases were detected without any false positive results. The above

workers generally preferred the immunofluorescent test over others, because a freshly prepared anti en was not every time required and moreover, the results were quickly obtained. Ambriose-Thomas (203) has reported that he obtained 412 positive results out of 412 cases tested by means of an immunofluorescence test. The results of the present study also substantiate the previous belief that it is a very accurate test procedure for detecting acute infections with liver involvement. The infection can be reliably diagnosed on the basis of a fairly high serum titres, which are generally obtainable in FA tests. Parekhar et al. (204) have successfully obtained titres as well, by using indirect immunofluorescence technic.

The ultraviolet absorption maxima of IgG and IgA were found near 278 nm. A minor hump at 290 nm indicated the presence of tyrosine, tryptophan and phenylalanine. The absorption maxima of IgM ranging from 250 - 270 nm suggested that this immunoglobulin contains more phenylalanine than IgG. These findings are correlating well with the previous work of Van Dalen et al. (205) on normal rabbit immunoglobulins. Their studies on amino acid composition indicated that IgM contained higher contents of alanine, phenylalanine, and histidine than IgG. Whereas lower contents of threonine, proline, tyrosine and lysine were found in IgM than in IgG. The fluorescence spectral studies also confirmed the presence of tryptophan, tyrosine and phenylalanine in the isolated immunoglobulins. No peak or hump was found near 305 - 310 nm, again indicating a higher tryptophan content of the molecule. The results of the present investigations are in agreement with the findings of Teale and Weber (206) on normal rabbit immunoglobulins.

The molecular weight of IgG from normal rabbit and human serum was estimated by different methods. This was found in the range of 140,000 - 160,000, though some recent estimations tend to indicate this value around 140,000 - 150,000 (Pain (207) and Harler et al. (208)). The molecular weight of antiamoeba IgG was found to be around 150,000, and it correlated well with the findings of the above mentioned workers. The Stoke's radius of IgG molecule, as calculated on the basis of its gel-filtration behaviour, was 4.613. This value is in good agreement with that of normal bovine IgG. Further, the frictional ratio which was found to be 1.318 suggested a globular nature of the molecule. But this might also have slight extension with two or three main globular regions linked by a flexible portion of the chain. Scoltock et al. (209) calculated the frictional ratio for normal rabbit IgG by the same method. Their value which came out as 1.47 suggested that IgG molecule is in the extended form with two or three main globular regions linked by a flexible portion of the chain. Yang (210) has calculated the frictional ratio of a globular protein and the value was ranging between 1.10 - 1.25. This value in the present study is higher than the one obtained by the above workers in normal rabbit IgG, confirming that IgG is a globular but, an extended molecule with an ellipsoidal or cylindrical shape. The diffusion coefficient was found to be 4.827×10^{-7} cm^2/sec. This observation also correlates well with the previous findings.

The carbohydrate contents of IgG, IgM and IgA were also measured. The hexoses present in all immunoglobulins were mannose

and galactose. Although their proportions varied in each immunoglobulin. The values obtained in the present study are correlating well with the findings of Fleischman et al. (211) and Chaplin et al. (212). The IgM and IgA were found to contain a considerably higher contents of carbohydrate than IgG (213,214).

From the previous studies, it is quite well known that humoral antibodies take part in protection against amebic infections. Present studies were also carried out to correlate the studies of humoral immune responses and make further attempts, to detect and evaluate the cell-mediated immune responses in the animals sensitized with E. histolytica antigens. Methods more commonly employed for the assessment of CMI are delayed skin hypersensitivity reactions, graft rejection studies, blast transformation, rosette formation and macrophage and leukocyte migration inhibition tests. Long and Pierce (215) in 1963 had indicated that immunity to a protozoan infection might be mediated by cells. The most common parameter employed for the evaluation of CMI response is the use of delayed skin hypersensitivity reactions. The appearance of skin hypersensitivity reactions have earlier been reported by Madison et al. (86,87) from experimental animals and, as well as from amebiasis patients. Similar results have also been reported by Miller and Scott (141), Kretschmer et al. (216), Savanat et al. (148) and Sherovitch and Scott (217). The investigations of Shegraith and Harinasuta (218) in guinea pigs have suggested that prolonged intestinal infection (maintained experimentally) may induce a state of hypersensitivity to invasion of

liver tissue by amebae. They have characterised two types of skin reactions in amebic infections:

I. The immediate type of skin reaction which is developed just after an intradermal challenge given to the sensitized guinea pigs. The test remains visible for 6 - 8 hrs. The reaction is developed with the injection of soluble antigen intradermally into an hyper immunized animal showing a fairly good level of precipitating antibodies. The animal responds by producing erythematous and oedematous reaction, reaching a peak at 3 - 8 hrs. The lesion is characterized by an intense filtration with polymorphonuclear cells.

II. The second type of reaction is the delayed hypersensitivity reaction, developing often between 10 - 24 hrs.

In the present investigations, the delayed type of reactions were used for assessing the CMI responses. The experimental animals developed a typical delayed skin reaction (127,128). Earlier, Leal (126) has also reported the appearance of a similar type of skin reaction in amebic patients. Kirkpatrick et al. (130) and Kretschmer et al. (139) carried out the skin reactions in sensitised animals. They have also tried to standardise the optimal antigen dose for using it as a diagnostic cutaneous test in humans. Unfortunately, they could not differentiate an immediate type of reaction with a true delayed type of hypersensitivity reaction. The findings of these studies clearly indicate that amoba antigen

preparation is capable of eliciting a cell-mediated immune response in guinea pigs. Lunde (143) has also carried out similar type of work. He was successful in developing a delayed skin hypersensitivity reaction in the 7th week with an optimal antigen dose of 20 ug used for challenging the animals.

Histologically, the earliest phase of the reaction is seen as a perivascular cuffing with mononuclear cells. This is followed by an extensive exudation of mono and polymorphonuclear cells. Predominantly, the lymphocytes and the cells of the monocyte-macrophage series are generally present at the site of the skin lesion. The presence of cellular immunity in amebiasis has been further demonstrated by macrophage inhibition tests (219,220). Amebin sensitive lymphoid cells, during incubation with specific antigen, secrete soluble substances (MIF) into the media which inhibit the migration of peritoneal exudate cells (219,221). This material is not detected in supernatants from lymph node cells when incubated with an unrelated antigen. This suggests that the production or release of MIF is only due to a specific immunologic reaction (221). The absence of histologic lesions in the liver of animals with MIF production further suggests the mediation of a cellular immune mechanism, which must also be participating in the defense mechanism of the host. The participation and the presence of cellular immunity in amebic infections have also been demonstrated by Gavanat et al. (148) in his lymphocyte blast transformation studies. He found that the transformation was of a specific nature. The cell-mediated immune responses varied from patient to patient or from individual to individual.

associated with the development of a well defined protective immunity. But the recovery from deeper tissue invasions like an acute amebic liver abscess, does provide an evidence of some protection against subsequent liver invasions. Although such a resistance against reinfection in extraintestinal cases does not seem to include subsequent intestinal infections (Sepulveda, B., personal communication). Epidemiological studies have suggested that the immune response is important in limiting the invasive activity of E. histolytica. It has also been suggested that an amebic disease must be preceded by a certain degree of immunodepression (227,228). In the absence of secondary bacterial invasion, the histological studies of amebic lesions of the intestine and liver have often shown a total absence of lymphocytic infiltration (229,230). This is a significant finding, perhaps suggesting a localised or generalized immunodepression state in active disease. In the initial stages of the disease, the detectable humoral antibodies do not appear to possess a potential population of specifically sensitised lymphocytes. This supports the hypothesis that subclinical invasion is likely to occur, unhampered. But progressive invasion of deeper tissue is presumably prevented or inhibited by a number of factors, one of which may quite possibly be immunological (231). In the preliminary stages, the amebic patients (and immunised animals) possess high level of globulins especially IgG and IgM. But a steady circulating population of sensitised lymphocytes, capable of responding to amebic antigen in vitro are generally not demonstrable in such cases. Although patients suffering from hepatic diseases do possess a circulating

In the present studies, the skin tests were found to develop after 2 weeks of immunization, while Lunde et al. (143) have shown the appearance of skin reactions in the 7th week. This discrepancy in time may be due to the differences in the route of inoculation and the immunization schedule. Guinea pigs in this study were intradermally immunized at different sites, and then, with small doses. The magnitude of the elicited immune response appeared to be quite high. Phillips et al. (222) have also made similar observations in their studies. They have reported that multiple small exposures are far more effective in inducing a lasting immunity than a single large exposure, even if, that single exposure is of much greater magnitude than the sum total of multiple small exposures. The results obtained from studies on peritoneal exudate cell migration by the capillary method look somewhat better than those from agarose plate technic. These differences may be due to the type of antigen employed. The impure and particulate antigens work better in capillary method than in agarose plates. Zabriskie and Falk (223), Maini et al. (224) and Corski (225), in their studies on BCG response, have also made similar observations.

From the findings of the present investigations and on the basis of previous work as well, it can be concluded that humoral and cell-mediated immune responses are elicited as a result of parasitic infections. In fact, both the humoral and cellular immune mechanisms operate simultaneously for the defense of the host against parasitic infections (Shaffer et al. (62) and Bran (226)). The recovery from acute intestinal diseases does not appear to be

associated with the development of a well defined protective immunity. But the recovery from deeper tissue invasions like an acute amebic liver abscess, does provide an evidence of some protection against subsequent liver invasions. Although such a resistance against reinfection in extraintestinal cases does not seem to include subsequent intestinal infections (Sepulveda, B., personal communication). Epidemiological studies have suggested that the immune response is important in limiting the invasive activity of E. histolytica. It has also been suggested that an amebic disease must be preceded by a certain degree of immunodepression (227,228). In the absence of secondary bacterial invasion, the histological studies of amebic lesions of the intestine and liver have often shown a total absence of lymphocytic infiltration (229,230). This is a significant finding, perhaps suggesting a localised or generalized immunodepression state in active disease. In the initial stages of the disease, the detectable humoral antibodies do not appear to possess a potential population of specifically sensitised lymphocytes. This supports the hypothesis that subclinical invasion is likely to occur, unhampered. But progressive invasion of deeper tissue is presumably prevented or inhibited by a number of factors, one of which may quite possibly be immunological (231). In the preliminary stages, the amebic patients (and immunised animals) possess high level of globulins especially IgG and IgM. But a steady circulating population of sensitised lymphocytes, capable of responding to amebic antigen in vitro are generally not demonstrable in such cases. Although patients suffering from hepatic diseases do possess a circulating

population of lymphocytes which are capable of responding *in vitro*. Such lymphocytes possess a substantially raised levels of specific antiamebic IgM and IgG. It can be concluded from the above findings, that primary amebic invasion of the bowel at clinical or subclinical level is in some way linked or associated with a certain degree of specific cellular immunodepression. Such an immune inhibition is possibly operating on the affarent pathway rather than inhibiting or destroying the effector cells. Because a central sensitization which may be inhibiting the effector cells is not apparently detectable. In latter stages of the disease the challenge becomes still greater, and of an altogether different nature, apparently due to the presence of high concentration of amoba antigen in the tissues and body circulation. This seems to be a compelling factor for giving rise to a typical cellular response. The above hypothesis corroborates the findings that a hepatic involvement is always accompanied by a fairly strong *in vitro* reaction of lymphocyte transformations to specific antigen. This response is suppressed locally as no small round cell response is in evidence, in liver abscess cases. Gershon (232) has described a similar mechanism in schistosomiasis. Accordingly, an immunodepression seems to be operative due to the induction of suppressor T cells by protein antigens. Similar phenomena have also been earlier described in some bacterial, viral and parasitic infections by Schwab (233).

C O N C L U S I O N

On the basis of results obtained in the present investigations, it can be concluded that E. histolytica antigen is sufficiently potent for the purpose of eliciting humoral and cell-mediated immune responses. The circulating antiamebic antibodies, both in patients and experimentally immunized animals, are detectable by means of a large number of antigen-antibody reactions like precipitin, indirect haemagglutination, bentonite slide flocculation, latex agglutination, immunodiffusion, immunoelectrophoresis and fluorescent antibody tests. During an amebic infection a primary response is followed by a typical secondary response. The magnitude of the secondary response is much higher than the primary response. The sequential appearance of antibodies, as evidenced by an experimental active immunization, is the production of IgM antibodies followed by IgG in that order. The persistence of these antibodies was detectable upto a period of 6 months after the primary immunization. The fractionation of circulating antibodies resolved into 3 peaks corresponding to IgG, IgA and IgM respectively on DEAE-cellulose column. On Sephadex G-200 column, the antibodies resolved into 3 peaks in the order of IgM, IgG and mixture of IgG, IgA and albumin. The results further indicate that IgG is the most abundant and reactive antiamebic immunoglobulin. The specific antiamebic activity as demonstrable by a large number of antigen-antibody tests was largely confined to the IgG fraction of the hyperimmune serum. Fahey (231) had also earlier made a similar observation that IgG is the most reactive immunogle-

bulin in the serology of amebiasis. Although, the possibility of some immunological activity associated with other immunoglobulins can not be altogether excluded.

Fraction IgA of the immune serum does not appear to have any specific role as an antiamebic immunoglobulin. Its level appeared to be, more or less, constant during the entire course of immunization. These observations on IgA are also in agreement with the findings of Abioye et al. (91). They did not find any significant relationship between serum levels of IgA and amebic infections, either.

The UV absorption maxima of IgG and IgA were found near 278 nm with a minor hump at 290 nm. These values indicate the presence of tyrosine, tryptophan and phenylalanine in IgG. The absorption of IgM ranged from 250 - 280 nm, suggesting a higher concentration of phenylalanine than in IgG. The amino acid composition studies further showed that IgM has higher concentrations of alanine, phenylalanine and histadine as compared to IgG. As also, the IgM has low contents of threonine, proline, tyrosine and lysine (205). The emission fluorescence maxima appearing at 350 nm for the isolated IgG, IgM and IgA also indicated the presence of tyrosine and tryptophan. No peak or hump was found near 305 - 310 nm, again indicating a higher tryptophan content of the molecule. The molecular weight was found to be 150,000, which is in good agreement with the previous findings of Pain (207) and Fahrer et al. (208) for normal IgG. Studies of Stoke's radius and frictional ratio of the IgG molecule confirmed that IgG is a globular but slightly extended

molecule, i.e. ellipsoidal or cylindrical. As far as CMI response is concerned, a delayed skin hypersensitivity reaction was generally detectable against E. histolytica antigen. The delayed hypersensitivity developed two weeks after primary immunization. The reaction after attaining its maximum size was subsequently found to subside. The histological investigations of skin reaction sites showed a cellular infiltration of polymorphonuclear and leukocyte cell-types. The migration of sensitized peritoneal exudate cells was strongly inhibited by ameba antigen in capillary tubes, as well as on agarose plates.

On the basis of present studies a few generalized statements can be made as follows:

1) The primary invasion of bowel at clinical or subclinical level is possibly associated with a certain degree of specific cellular immunosuppression. This seems to be on the afferent pathway rather than straightway inhibiting, or destroying the effector cells. The absence of any protection against reinfection during early stages can quite well be explained on the basis of the above hypothesis.

2) In the early stages of the disease, some such factors are also being released which specifically inhibit the synthesis of T_{10} lymphocytes. Since T_{10} lymphocytes are directly responsible for the appearance of DH reactions responsible for providing protection, therefore, their inhibition results in the non-availability of any immune protection during early stages.

3) Further efforts should therefore be directed to test the inhibition, or otherwise, of T_{10} lymphocytes during the course of the disease. Efforts should also be made to detect the presence of more unknown factors like cytotoxic T lymphocytes or killer L lymphocytes, which are also capable of actively inhibiting the synthesis of specific lymphocytes responsible for protection.

CHAPTER - VI

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APPENDIX

Presentation and Publications.

1. Ali Sher and Sohail Ahmad. Characterization of immune sera in amebiasis. Proc. & Abs. Soc. Biol. Chemists (India). 32: 14 (1973).

2. Ali Sher and Sohail Ahmad. Detection of antiamoebic antibodies in sequential sera samples. Proc. & Abs. I Ann. Conf. Ind. Immunol. (1973).

3. Sohail Ahmad, Ali Sher, Mashiat J. Chilliqui, Shamsuddin Bisati and Siraj Hussain. Detection of Entamoeba histolytica trophozoites in peripheral blood. Ind. J. Parasitol. 1(2): 129 (1977).

4. Ali Sher and Sohail Ahmad. Detection and characterization of humoral antibodies from amoebic immune sera. Ind. J. Exp. Biol. 15: 1083 (1977).

5. Ali Sher and L. Muller-Berat. In vitro stimulation of colony forming cells (CFC) to granulomonocytopoiesis by PHA in mice. Presented at the Sixth Annual Conference of the International Society of Experimental Hematology (Switzerland), August (1977).

6. N. Muller-Berat, Ali Sher and D. Marin. Hemopoietic stem cells and colony stimulating activity (CSA) after PHA in vivo in C3H/HeJ Mice. Presented at the Sixth Annual Conference of the Society of Experimental Hematology (Switzerland), August (1977).

7. Ali Sher, Sohail Ahmad and L. Muller-Berat. Macrophage migration inhibition as a correlate of cell-mediated immunity against Entamoeba histolytica. To be presented at the Asian Congress of Parasitology, Bombay, on Feb. 23 - 26 (1978).